UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

Estudio de la Participación de MGL1 en la Activación y Funcionalidad de Macrófagos en la Infección por *Trypanosoma cruzi*

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTOR EN CIENCIAS BIOMÉDICAS

PRESENTA:
MARIO ALBERTO TONATHIU RODRÍGUEZ HERNÁNADEZ

DIRECTOR DE TESIS
DRA. MIRIAM RODRIGUEZ SOSA
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA
COMITÉ TUTOR
DRA BERTHA ESPINOZA GUTIÉRREZ
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS
DRA. MARTHA LEGORRETA HERRERA
FACULTAD DE ESTUDIOS SUPERIORES ZARAGOZA

LOS REYES IZTACALA, ESTADO DE MEXICO
AGOSTO, 2020

Universidad Nacional Autónoma de México
UNAM
Biblioteca Central
Dirección General de Bibliotecas de la UNAM

UNAM – Dirección General de Bibliotecas
Tesis Digitales
Restricciones de uso

DERECHOS RESERVADOS ©
PROHIBIDA SU REPRODUCCIÓN TOTAL O PARCIAL

Todo el material contenido en esta tesis esta protegido por la Ley Federal del Derecho de Autor (LFDA) de los Estados Unidos Mexicanos (México).

El uso de imágenes, fragmentos de videos, y demás material que sea objeto de protección de los derechos de autor, será exclusivamente para fines educativos e informativos y deberá citar la fuente donde la obtuvo mencionando el autor o autores. Cualquier uso distinto como el lucro, reproducción, edición o modificación, será perseguido y sancionado por el respectivo titular de los Derechos de Autor.

Dedicatoria:

A mi Jaque por ser mi amiga, mi compañera, mi amada; por su cariño y apoyo "in"-condicional durante toda nuestra vida de pareja y en especial durante este proceso; por estar conmigo en todo momento ¡muchas gracias!. A Astrid, por ser mi motivacion en cada cosa que hago, por estar y aguantarme en las buenas y sobre todo en las malas.

A mis padres Mario y Loudres por haberme dado la vida y permitirme el haber llegado hasta este momento tan importante de mi formación profesional. Ustedes quienes me enseñaron con su ejemplo dos lecciones de esta vida, la primera fue la dedicacion: que incluso la tarea más grande se puede lograr si se hace un paso a la vez, y segundo la perseverancia: mantenerme constante en un proyecto ya comenzado, aún cuando las circunstancias sean adversas o los objetivos no puedan ser cumplidos. Estas dos leccionees son las mas dificiles, he de confesar, todavia las estoy aprendiendo.

A mi hemana Miriam por apoyarme cuando más la he necesitado, por extender su mano en momentos difíciles y por el amor brindado cada día, de verdad mil gracias hermanita, siempre te llevo en mi corazón. A mis sobrinos Memo, Gabo y a mi cuñado Pepe, por ser una parte muy importante de mi familia.

A mi tia Tere y Manolo por todo el tiempo desde mi existencia, desde mi niñez hasta este momento, por compartir su buen humor y por su compañía.

A Pável, a mi amigo, a mi primo, a mi hermano.

A Andy y a mi madrina Magos, quienes siempre ha estado en mi vida

A Andrea y Fanny mis primas quienes me han dado una gran cantidad de recuerdos y alegrias.

A Vicky por ser mi amiga desde hace mucho tiempo, gracias por tantos años y por los futuros.

Agradecimiento:

Primeramente, me gustaría agradecer a la persona que me ha influenciado mas en mi vida, desde un punto de vista académico y humano, a mi madre académica la Dra. Miriam Rodriguez Sosa, es y será siempre importante en mi vida personal y profesional, personal por enseñarme la compasión y tolerancia hacia las demás personas, y enseñarme que todas las personas tienen un talento y el trabajo del líder es encontrarlo y potenciarlo, las debilidades se tienen que trabajar mas arduamente. Además, profesionalmente me enseño a que siempre se tiene que dar el mejor esfuerzo que cada cosa que realices ya sea trabajo manual o escrito (incluso en un correo electrónico). Quiero agradecerle por cada detalle y momento dedicado para aclarar cualquier tipo de duda, y enseñarme en cada platica, discurso y lección. Además, por haberme dado la oportunidad de ser su alumno y por haberme permitido mi desarrollo de posgrado en su laboratorio.

A mi asesora Martha Legorreta, por su apoyo en este proceso y asesoramiento en mi doctorado.

A mi asesora Bertha Espinoza, quien siempre me dijo palabras de reflexion y superacion.

A Alicia por haberme ayudado en el proceso de mi doctorado, por haberme orientado y apoyado en todos los momentos que necesité sus consejos, ademas de ser una gran amiga.

A Imelda por el apoyo que me has dado, por tus sus consejos y correcciones; y por tu amistad.

AVictor Hugo (Vic) por ser un gran amigo, compañero, por todas esas platicas que tuvimos, y por todas las horas en el gimnacio, lo difrute mucho.

A Alexia, por nuestras experiencias juntos, conviviendo, aprendiendo de cada aventura y risa, me has permitido ser tu amigo, mil gracias por todo lo que me das.

A Danae por todo lo que hemos pasado está lleno de cosas hermosas, pruebas, así como alegrías y tristezas, muchas gracias por todo.

A Oscar Nieto un amigo al cual le he aprendido la humildad, humandidad y el trato hacia los demas; siendo esta una delas mas importantes lecciones en mi vida. Espero que nuestra amistad cresca con los años.

A Thalia por haberme escuchado en el proyecto de *T. cruzi*-cancer (que ya lo habia comentado previamente con varios compañeros sin haber recibido respuesta); y al siguiente dia empezamos a trabajar, ¡somos un buen equipo!

A Bethsaida, por su amistad y compañerismo

A Martita, Octavio, Ulises, Oscar Iliesca, Raziel y Andres, que con sus palabras, trabajo y amistad hicieron que pasara un muy agradable el timpo en el laboratorio.

De igual manera mis agradecimientos a la UNAM, al Doctorado en Ciencias Biomédicas y a todo los profesores que contribuyeron en mi educacion de Doctorado por su enseñanza, paciencia y dedicación.

Este Trabajo se realizó en el laboratorio 5, “Inmunidad Innata”, en la Unidad de Biomedicina (UBIMED) de la Faculta de Estudios Superiores Iztacala

El sustentante: Mario Alberto Tonathiu Rodríguez Hernández fue becario del Consejo Nacional de Ciencia y Tecnologia (CONACyT), No de becario (CVU): 550200

Voto Aprobatorio

Cd. Universitaria, Cd.Mx, a 10 de junio de 2020.

C.P. AGUSTÍN MERCADO
Director de Certificación y Control Documental
Escolar, UNAM
P r e s e n t e.

Estimado C.P. Mercado:

Después de haber evaluado la Tesis titulada **Estudio de la participación de MGL1 en la activación y funcionalidad de macrófagos en la infección por *Trypanosoma cruzi*** del alumno **Mario Alberto Tonathiu Rodríguez Hernández**, con número de cuenta **30126897-6**, inscrito en el Doctorado en Ciencias Biomédicas y aspirante al grado de Doctor en Ciencias, considero que ésta reúne los requisitos suficientes y la acepto para ser presentada en el examen de grado.

Por lo anterior, emito mi **VOTO APROBATORIO**.

Agradezco de antemano la atención que se sirva prestar a la presente.

Atentamente

Presidente: Dra. Ingeborg Dorothea Becker Fauser

Voto Aprobatorio

Cd. Universitaria, Cd.Mx, a 10 de junio de 2020.

C.P. AGUSTÍN MERCADO
Director de Certificación y Control Documental
Escolar, UNAM
P r e s e n t e.

Estimado C.P. Mercado:

Después de haber evaluado la Tesis titulada **Estudio de la participación de MGL1 en la activación y funcionalidad de macrófagos en la infección por *Trypanosoma cruzi*** del alumno **Mario Alberto Tonathiu Rodríguez Hernández**, con número de cuenta **30126897-6**, inscrito en el Doctorado en Ciencias Biomédicas y aspirante al grado de Doctor en Ciencias, considero que ésta reúne los requisitos suficientes y la acepto para ser presentada en el examen de grado.

Por lo anterior, emito mi **VOTO APROBATORIO**.

Agradezco de antemano la atención que se sirva prestar a la presente.

Atentamente

Secretario: Dra. Miriam Rodríguez Sosa

Universidad Nacional Autónoma de México
Depto. de Microbiología y Parasitología
Facultad de Medicina. Edificio A, 2do Piso.
Ciudad Universitaria. México, D.F. 04510
Tel: 56 23 23 58, Fax: 56 23 23 57
Correo e: landap@unam.mx

Cd. Universitaria, Cd.Mx, a 10 de junio de 2020.

C.P. AGUSTÍN MERCADO
Director de Certificación y Control Documental
Escolar, UNAM
P r e s e n t e.

Estimado C.P. Mercado:

Después de haber evaluado la Tesis titulada **Estudio de la participación de MGL1 en la activación y funcionalidad de macrófagos en la infección por *Trypanosoma cruzi*** del alumno **Mario Alberto Tonathiu Rodríguez Hernández**, con número de cuenta **30126897-6**, inscrito en el Doctorado en Ciencias Biomédicas y aspirante al grado de Doctor en Ciencias, considero que ésta reúne los requisitos suficientes y la acepto para ser presentada en el examen de grado.

Por lo anterior, emito mi **VOTO APROBATORIO**.

Agradezco de antemano la atención que se sirva prestar a la presente.

Atentamente

Vocal: Dr. Abraham Landa Piedra

Voto Aprobatorio

Cd. Universitaria, Cd.Mx, a 10 de junio de 2020.

C.P. AGUSTÍN MERCADO
Director de Certificación y Control Documental
Escolar, UNAM
P r e s e n t e.

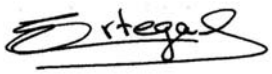
Estimado C.P. Mercado:

Después de haber evaluado la Tesis titulada **Estudio de la participación de MGL1 en la activación y funcionalidad de macrófagos en la infección por *Trypanosoma cruzi*** del alumno **Mario Alberto Tonathiu Rodríguez Hernández**, con número de cuenta **30126897-6**, inscrito en el Doctorado en Ciencias Biomédicas y aspirante al grado de Doctor en Ciencias, considero que ésta reúne los requisitos suficientes y la acepto para ser presentada en el examen de grado.

Por lo anterior, emito mi **VOTO APROBATORIO**.

Agradezco de antemano la atención que se sirva prestar a la presente.

Atentamente



Vocal: Dr. Enrique Ortega Soto

Voto Aprobatorio

Cd. Universitaria, Cd.Mx, a 10 de junio de 2020.

C.P. AGUSTÍN MERCADO
Director de Certificación y Control Documental
Escolar, UNAM
P r e s e n t e.

Estimado C.P. Mercado:

Después de haber evaluado la Tesis titulada **Estudio de la participación de MGL1 en la activación y funcionalidad de macrófagos en la infección por *Trypanosoma cruzi*** del alumno **Mario Alberto Tonathiu Rodríguez Hernández**, con número de cuenta **30126897-6**, inscrito en el Doctorado en Ciencias Biomédicas y aspirante al grado de Doctor en Ciencias, considero que ésta reúne los requisitos suficientes y la acepto para ser presentada en el examen de grado.

Por lo anterior, emito mi **VOTO APROBATORIO**.

Agradezco de antemano la atención que se sirva prestar a la presente.

Atentamente

Vocal: Dra. Martha Torres Rojas

Índice

Abreviaturas

(^{3}H)-TdR	Timidina tritiada
AgTc	Antígeno de *T. cruzi*
APC	Aloficocianina
CDs	Células dendríticas
CLRs	Receptores tipo lectina dependientes de calcio
CPAs	Células presentadoras de antígenos
CRD	Dominios de reconocimiento de carbohidratos
DC-SIGN	Adhesión específica intercelular de molécula-3-grabbing no integrina de las células dendríticas
FCS	Suero fetal bovino
FITC	Fluoresceina
Gal	Galactosa
GalNAc	N-Acetil galactosa
GIPLs	Glicolinositolfosfolípidos
GPI	Glicosilfosfatidilinositol
hMGL	*MGL Humano*
IFN-γ	Interferón-gama
IFN-γR	Receptor de IFN-γ
IL	Interleucina
IMF	Intensidad media de fluorescencia
iNOS	óxido nítrico sintetasa inducible
ITAM	Inmunoreceptor basados en la activación de tirosina

Le^A	Lewis A
Le^x	Lewis X
MGL	lectina de galactosa de macrófago
mMGL	MGL murino
Mφ	Macrófagos
MR	Receptor de manosa
NO	Óxido nítrico
PE	Ficoeritrina
PECs	Células totales de la cavidad peritoneal
RNI	Intermedios reactivos de nitrógeno
ROS	Especies de oxígeno reactivo
SEAs	Antígeno soluble de huevos de *Schistosoma mansoni*
T. cruzi	*Trypanosoma cruzi*
TLR	Receptor Tipo Toll
TNF-α	Factor de necrosis tumoral-alfa

Abstract

The macrophage galactose type C lectin receptor (MGL) is a receptor expressed in macrophages (Mφ) and dendritic cells (CDs). mMGL is specific for terminal galactose (Gal) and terminal N-Acetylgalactose (GalNAc) residues expressed on the surface of *T. cruzi*, however, the role of MGL in immune function and activation in Mφ against infection by *T. cruzi* remains unknown. In the present work it is shown that the Mφ MGL1$^{-/-}$ infected with *T. cruzi in vitro* had a greater number of internal parasites than the WT Mφ. Likewise, the internal parasites of Mφ MGL$^{-/-}$ show higher viability, with a lower production of nitric oxide (NO), reactive oxygen species (ROS), interleukin (IL) -12 and tumor necrosis factor (TNF) -α in comparison with the Mφ WT. In addition, the presence of mMGL is associated with a lower expression of TLR-2 and TLR-4. Phosphorylation of NF-κB through *T. cruzi* antigens (AgTc) induccions was reduced in Mφ mMGL$^{-/-}$. Importantly, protein levels of the kinase regulated by extracellular phosphorylated signals 1, 2 (p-ERK1,2), p-c-jun and p-nuclear factor (NF) -kB p65 were significantly reduced and activation of the inflammasome NLRP3 and IL-1β were reduced in MGL1$^{-/-}$ Mφ treated with Ag*Tc* compared to WT Mφ treated with Ag*Tc*. In addition, mMGL played a key role in the expression of MHC-II, favoring the activation of antigen-specific cells for T. cruzi infection. Our data reveal a previously mentioned importance of MGL in the activation of Mφ, through the modulation of the signaling pathways of the inflammasome NFκB, ERK1,2 and NLRP3, and the development of protective immunity against experimental infection by *T. cruzi*.

Resumen

El receptor de galactosa tipo lectina C de macrófago (MGL) es un receptor expresado en macrófagos (Mφ) y células dendríticas (CDs). MGL reconoce residuos de galactosa (Gal) y N-Acetilgalactosa terminal (GalNAc) expresados en la membrana de *T. cruzi*, sin embargo, se desconoce el papel inmune que desempeña MGL en la función y activación de los macrófagos (Mφ) contra la infección por *T. cruzi*. En el presente trabajo se muestra que los Mφ deficientes para MGL1 infectados con *T. cruzi in vitro* tuvieron un mayor número de parásitos internos que en comparación con los Mφ WT. Así mismo, los parásitos internos de los Mφ MGL1$^{-/-}$ muestran mayor viabilidad relacionados con una menor producción de óxido nítrico (NO), especies de oxígeno reactivo (ROS), interleucina (IL) -12 y factor de necrosis tumoral (TNF) -α en comparación en los Mφ WT. Además, la presencia de MGL1 es asociado con una menor expresión de TLR-2 y TLR-4. La fosforilación de NF-κB a través de los antígenos de *T. cruzi* se redujo en los Mφ MGL1$^{-/-}$. Es importante destacar que los niveles de proteína de la quinasa regulada por señales extracelulares fosforiladas 1, 2 (p-ERK1,2), p-c-jun y el factor p-nuclear (NF)-κB p65 se redujeron significativamente y la activación del inflamasoma NLRP3 e IL-1β se redujo en Mφ MGL1$^{-/-}$ tratados con *Tc*Ag en comparación con Mφ WT tratado con *Tc*Ag. Además, mMGL desempeñó un papel clave en la expresión de MHC-II, favoreciendo la activación de células específicas de antígeno para la infección por *T. cruzi*. Nuestros datos revelan que MGL1 es importante en la activación de Mφ, a través de la modulación de las vías de señalización del inflamasoma c-jun, NFκB, ERK1,2 y NLRP3, y el desarrollo de inmunidad protectora contra la infección experimental por *T. cruzi*.

Introducción

El sistema inmune está provisto de componentes celulares y humorales con la capacidad de proteger al huésped contra patógenos, sustancias extrañas y daño tisular. Los macrófagos (Mφ) son un grupo de células importantes en la eliminación de parásitos, estas células provienen de monocitos que al ser reclutados a los sitios de infecciones o lesiones se diferencian en Mφ, estas células juegan un papel decisivo en respuestas a bacterias intracelulares [1], y representan la primeria línea de defensa de la inmune innata. Las funciones efectoras de los Mφ constan de la producción de moléculas tóxicas, como el óxido nítrico (NO), intermedios reactivos del oxígeno (ROS), así como la producción de citocinas [2, 3]. Sin embargo, los parásitos han evolucionado para contrarrestar las estrategias del sistema inmune del huésped para asegurar la supervivencia dentro de este. El parásito protozoario intracelular *Trypanosoma cruzi* es el agente causante de la enfermedad de Chagas. Esta infección ocurre principalmente en América Latina, no obstante en la pasada décadas se ha detectado en Estados Unidos de América, Canadá, países europeos y países del Pacífico occidental [4], convirtiéndose esta enfermedad un nuevo desafío mundial.

Trypanosoma cruzi (T. cruzi)

T. cruzi pertenece a la clase kinetoplastidae, que se caracterizan por ser grupo de protozoos parásitos con presencia de un flagelo en al menos una etapa del ciclo de vida; un inusual ADN mitocondrial expandido llamado kinetoplasto y la presencia de variantes morfológicas a través del ciclo de vida. Estos organismos están estrechamente relacionados con los euglenoides, siendo parásitos en plantas, insectos y vertebrados [5]. Los protozoarios de la familia Trypanosomatidae son agentes de enfermedades parasitarias que tienen una alta incidencia y un impacto económico negativo en los países en desarrollo. La Enfermedad de Chagas es

causada por el parasito protozoario flagelado *T cruzi*; las vías de transmisión son: transfusiones sanguíneas [6], trasplantes de órganos infectados [7], transmisión oral por alimentos contaminados [8], congénita [9] y la manera natural de infección es vectorial la cual se produce en América latina. Los vectores son insectos de la subfamilia Triatominae (chinches) portadores del parásito causante de la enfermedad. Los triatomíneos salvajes pueden dar lugar a nuevos casos de enfermedad de Chagas humana [10]. Una característica específica de los tripanosomatidos es el cambio de su morfología durante su ciclo de vida, implicando la aparición de etapas de división e infecciosas, a través de un proceso descrito como diferenciación o transformación. *T. cruzi* tiene un ciclo de vida digénico de hospedadores vertebrados e invertebrados [11].

Epidemiología de *T cruzi*

Se estima que en el mundo hay entre 6 y 7 millones de personas infectadas en el mundo por *T. cruzi*, la mayoría de ellas en América Latina. Inicialmente, la enfermedad de Chagas estaba confinada a la Región de América Latina, pero se ha propagado a otros continentes, la OMS en el año de 2005, la reconocio en el año como una enfermedad tropical desentendida. [12]. El desplazamiento y la migración de individuos de áreas endémicas a no endémicas presentan un riego en la propagación de esta enfermedad. Por esta razón, se estiman 300,000 personas infectadas en Estados Unidos [13]. Así mismo, en Europa se estiman entre 59,000 y 108,000, con un mayor número en España e Italia [14]. La presencia de triatominos en la residencia es un factor de riesgo para adquirir *T. cruzi*. Un estudio realizado en 990 casas rurales en la región sur del Estado de México se encontró que un 28 % de los triatominos portaban *T. cruzi*. Los vectores están adaptados para vivir y reproducirse en el ambiente doméstico, mostrando así un riesgo potencial de transmisión de la enfermedad de Chagas a los seres humanos [15].

Fases de la infección

Cuando el individuo se infecta con *T. cruzi* puede cursar por dos fases: la primera es la fase aguda, se caracteriza por la presencia del parasito en circulación, además, algunas veces se presenta inflamación en el sitio de inoculación del parasito, esta fase representa el primer contacto entre el parásito y el huésped. La segunda fase es la fase crónica, se caracteriza por la ausencia del parasito en circulación. La fase crónica se subdivide en dos fases; la primera es asintomática, esta puede durar meses incluso años; la segunda es sintomática en la cual se presenta en aproximadamente de 10-30 % de las personas que adquieren esta enfermedad. En esta etapa pueden presentar enfermedades en el tracto digestivo como mega-esófago y mega-colon, y/o presentar cardiopatías relacionadas con una gran cantidad de nidos de amastigotes en el tejido cardiaco. La destrucción del miocardio (característica de las formas clínicas cardiacas más severas de la enfermedad de Chagas), es causada por una reacción inflamatoria progresiva multifocal asociada a lesión del endotelio vascular, vaso espasmo y reducción del flujo sanguíneo [16]. En la fase aguda se desencadena la respuesta inmunológica, dichos estos eventos inmunológicos influyen en el desarrollo de la respuesta protectora y la eliminación del parasito; si este parasito prevalece, se desarrolla la fase crónica; En esta fase, *T. cruzi* se dirige a diferentes tejidos del huésped como los tejidos linfoides periféricos y centrales[17].

Ciclo de vida de *Trypanosoma cruzi*

T. cruzi presenta un ciclo de vida complejo que implican etapas de desarrollo dentro de los huéspedes vertebrados e invertebrados. Así como en el torrente sanguíneo y dentro de las células huésped vertebradas.

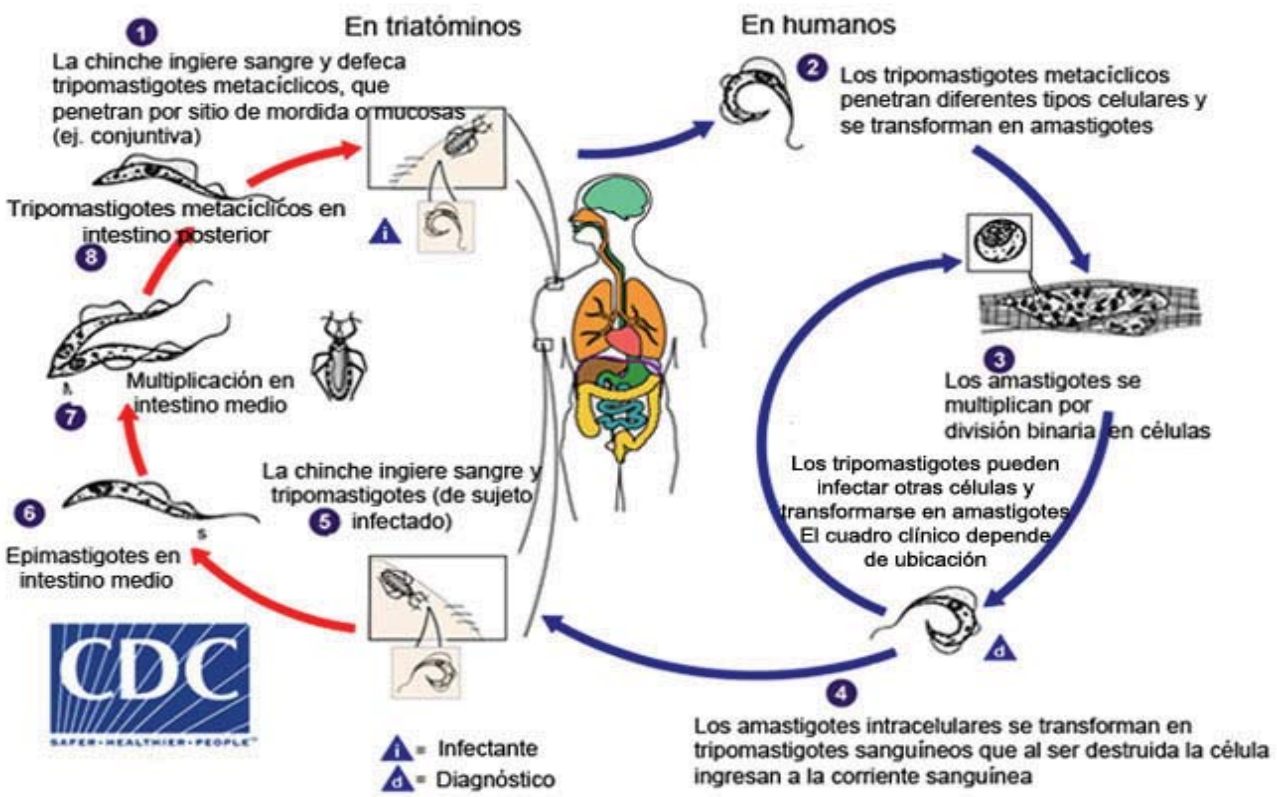


Figura 1. Ciclo de vida de *Trypanosoma cruzi*. (https://www.cdc.gov/parasites/chagas/es/index.html)

Triatomino

El ciclo comienza en el Triatomino de la familia Reduviidae que realiza hematofagia del vertebrado infectado con las formas de trypomastigotes sanguíneos que circulan en el torrente sanguíneo. Una vez ingerido, la mayoría de los trypomastigotes se lisan en el estómago del insecto [18]. Los trypomastigotes sobrevivientes se transforman, en pocos días, ya sea en estadios esféricos (conocidos como esferomastigotes) o en estadios epimastigotes. Los epimastigotes migran al intestino del insecto donde se dividen por fisión binaria y se adhieren a las membranas perimicrovillar presentes en las células del intestino medio posterior [19, 20]. La adhesión es importante para desencadenar el proceso de transformación de los epimastigotes no infecciosos en tripomastigotes infecciosos (conocidos como tripomastigotes metacíclicos). El proceso de adhesión de epimastigotes a las membranas perimicrovilares implica la participación de glicoconjugados expuestos a la superficie. Los glicolinositolfosfolípidos (GIPLs) de superficie

del parásito que están involucrados en el proceso de unión [20]. En la mayoría de las regiones posteriores del intestino y en el recto, muchos epimastigotes se separan de la superficie intestinal y se transforman en formas trypomastigotes metacíclicos que luego se liberan junto con las heces y la orina.

Mamífero

Los trypomastigotes metacíclicos infectan a los mamíferos por inoculación directa, o de manera indirecta por el contacto de heces y la orina en la mucosa ocular o la piel lesionada por el prurito ocasionado por la picadura del triatomino. Una vez dentro del huésped vertebrado, los trypomastigotes metacíclicos invaden las células en el sitio de inoculación (por ejemplo, fibroblastos, macrófagos y células epiteliales). El ciclo de vida intracelular de *T. cruzi* implica varios pasos como, el reconocimiento de la célula huésped a *T. cruzi,* la internalización del parasito a la célula y la formación a vacuola endocítica conocida como vacuola parasitófora, [21]. Ahora contenidos en la vacuola parasitófora ácida, la protección del parásito se da por la expresión de trans-sialidasas en la superficie del parasito. El mecanismo para el escape vacuolar es lisosoma-pH dependiente, además de la secreción de una proteína formadora de poro parecida a la proteína del complemento 9 conocida como TcTOX, además del factor lítico LYT1. El pH ácido de la vacuola mediante fusión lisosómica, LYT1 y/o TcTOX, son capaces de promover la lisis de membrana vacuolar que permite la entrada citoplásmica. El trypomastigotes cambia a su fase replicativa conocida como amastigote, los cuales reingresan al ciclo celular y se someten a ocho a nueve rondas de replicación antes la diferenciación trypomastigotes sanguíneos. Posteriormente lisan la célula huésped para viajar por torrente sanguíneo para infectar a otras células [22] (Figura 1).

Membrana citoplasmática de *T. cruzi*

Existen moléculas antigénicas ancladas en la superficie de la membrana citoplasmática de *T. cruzi*, dentro estas se encuentran el glicosilfosfatidilinositol (GPI) (Figura 2) [23]. Los GPI en *T.*

cruzi juegan un papel activación del sistema inmune innato del hospedero durante la infección, provocando la síntesis de citocinas y quimiocinas proinflamatorias, la generación de intermedios reactivos del nitrógeno (RNI), así como la expresión de moléculas de adhesión por los Mφ del huésped y las células endoteliales [23].La mayoría de GPIs (libres o asociadas a proteínas) tienen en común una estructura de núcleo hidrófilo con un motivo conservado definido por la secuencia Mana1-2Mana1-6Mana1-4GlcNa1-6myo-inositol-1-PO_4. [23].

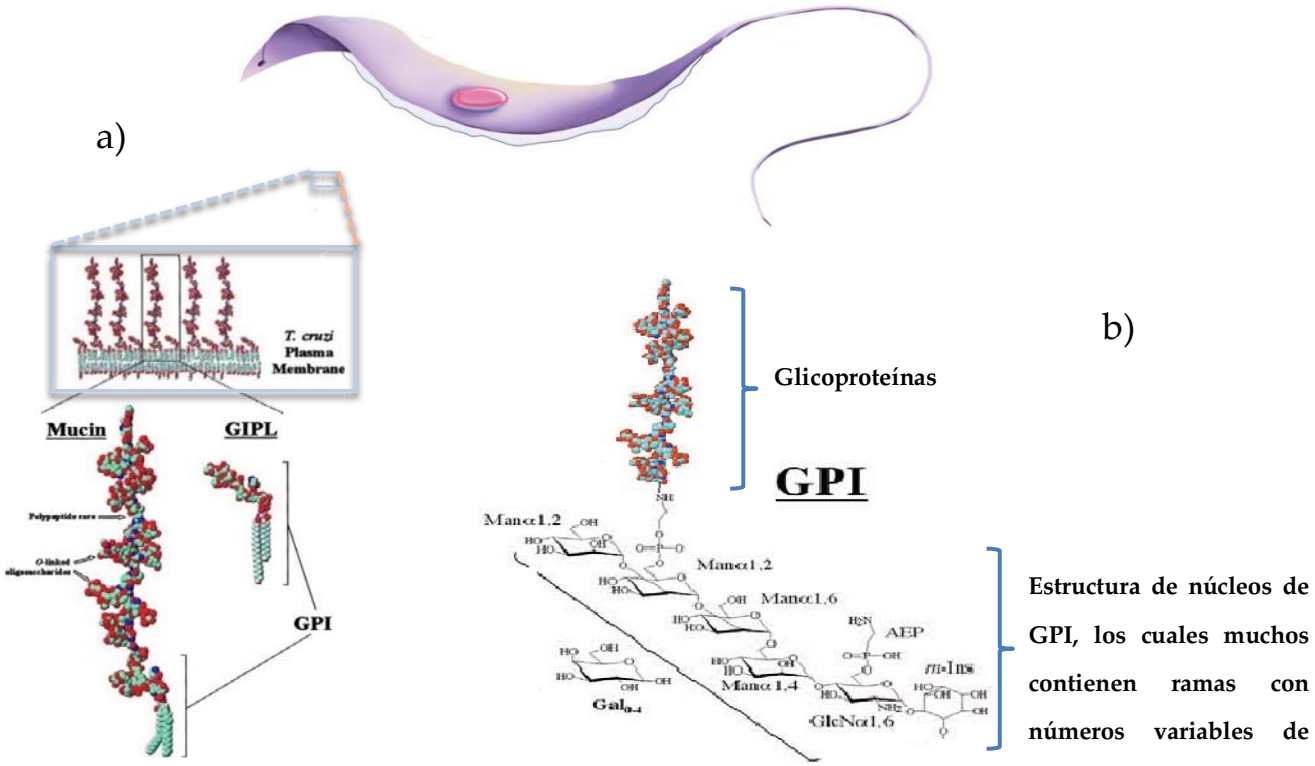


Figura 2. Moléculas principales ancladas de la membrana plasmática de *T. cruzi*. a) Mucina; (la glicoproteína del parásito) GIPL; glicoinositolfosfolípido. Dependiendo de la etapa de desarrollo, el número de estas moléculas varía de 10^6 a 10^7 cubriendo del 60-80% de la membrana. Átomos de carbono (Azul claro), hidrógeno (blanco), oxígeno (rojo) y nitrógeno (azul oscuro indican). Por simplicidad, los átomos de hidrógeno sólo se muestran en el resto lipídico de la estructura GPI (Modificado [23]). b) Estructura primaria de las principales moléculas de *T. cruzi* GPI ancladas. GPI de *T. cruzi* (modificado [24]).

Respuesta inmune en la Infección con *T. cruzi*

La forma Trypomastigote sanguíneo pertenece a la fase aguda infecciosa de *T. cruzi*, este invade los Mφ y otros tipos de células [25]. Los receptores presentes en las células de la inmunidad innata, como Mφ y CDs reconocen moléculas presentes en la membrana citoplasmática de *T. cruzi,* provocando una respuesta inflamatoria en tejidos infectados, controlando la replicación y propagación de *T. cruzi*. Se ha demostrado que la infección por *T. cruzi* induce a la producción de citocinas proinflamatorias en monocitos y macrófagos tanto en humanos como en el modelo murino de infección [26-28]. La producción de IFN-γ, IL-12 y TNF-α favorece el control de la parasitemia de *T. cruzi* en la fase aguda en el modelo murino [24]. La colonización de *T. cruzi* en el huésped da lugar a una respuesta aguda sistémica del hospedero que el parásito debe superar. *T. cruzi* interfiere con la función de presentación de antígeno de las células dendríticas a través de una acción sobre el receptor del ácido sialico de tipo Ig, induciendo la supresión de las respuestas de células T $CD4^+$ [17]. GPI-ancladas a mucinas en la membrana citoplasmática de *T. cruzi,* son glicoproteínas de los trypomastigotes, estas son responsables de la activación del Mφ. La activación del Mφ es inducida por GPI-mucinas purificadas de epimastigotes y trypomastigotes de tejidos; estimulando la síntesis citocinas proinflamatorias y óxido nítrico (NO) [29]. Además, la infección por *T. cruzi* en Mφ provocó un ambiente extremadamente oxidativo dentro del fagolisosoma, para la eliminación del parasito[30].

El NO es una molécula con propiedades citotóxicas contra parásitos intracelulares, ésta interactúa con oxígeno y anión superóxido para producir especies de nitrógeno reactivo (ROS), los cuales promueven la eliminación de parásitos intracelulares. El NO se produce por la enzima inducible de óxido nítrico sintetasa (iNOS) que cataliza la conversión de L-arginina a L-citrulina produciendo NO a partir del átomo terminal de nitrógeno del grupo guanidino de la arginina en las células inflamatorias activadas. El promotor iNOS humano es activado por NF-κB y Stat1, estos factores de transcripción son activados por la vía de los TLRs y del receptor de IFN-γ (IFN-

γR) respectivamente. En células RAW 264.7 la inhibición de las expresiones iNOS, IL-6, IL-1β y TNF-α se deben a una regulación negativa de la activación de NF-κB a través de supresión de la fosforilación de IKK y MAP quinasa (p38, ERK1/2 y JNK) [31, 32]. Así mismo, se han mostrado que ERK1/2 regula positivamente la producción de NO. La fosforilación de p42/44 (ERK1/2) MAPK (ERK-1/2), aumenta notablemente en las células con iNOS. Mostrando que NO producido activa la cascada de señalización ERK-1/2, que regula la proliferación del VSMC y la regulación positiva de la expresión p21 por NO [33]. Estudios de la red de expresión por ARN de c-jun por microarreglos de WT y Mφ de ratones condicionales para c-Jun han demostrado la participación en c-jun en unos 700 genes. Además, el enriquecimiento de la ontología genética y el análisis de conglomerados revelaron varias redes asociadas a genes pro y antiinflamatorios, como la proliferación de células inmunes, la regulación de la producción de citocina , la señalización de la respuesta inmune y la respuesta a la hipoxia, confirmaron que c-Jun es un factor de transcripción central durante la activación de Mφ [34]. La etapa aguda de la infección de *T. cruzi* es seguida por la etapa indeterminada, ésta es asociada con un perfil de citocinas antiinflamatorias como IL-10 [35].

Toll Like Receptors

Los receptores tipo toll (TLRs) forman parte de la inmunidad innata del huésped contra *T. cruzi,* son críticos para eliminación de *T. cruzi* [36-38]. Los TLRs desempeñar un papel importante en la regeneración de los tejidos [39, 40], así como en la cardio protección en sepsis experimental [41, 42]. En células presentadoras de antígenos (CPAs) TLR-2 participa como regulador positivo de los mecanismos de defensa durante las primeras etapas de la infección de *T. cruzi*. Se ha demostrado que TLR2 regula la activación de Rab5, que es necesario para la internalización de *T. cruzi* en Mφ [43]. Además, GPI de *T. cruzi* que se encuentra unido a glucoproteínas de tipo mucina (glucosilfosfatidilinositol-mucina) en la membrana de *T. cruzi,* es agonista del heterodímero TLR2-TLR6. Por otra parte, glicosilfosfatidilinositol lipido (GPIL) de *T. cruzi*, son

reconocidos por el complejo TLR4-MD2 produciendo IL -12, TNF-α y NO por Mφ (fig. 3) [7, 43-46].

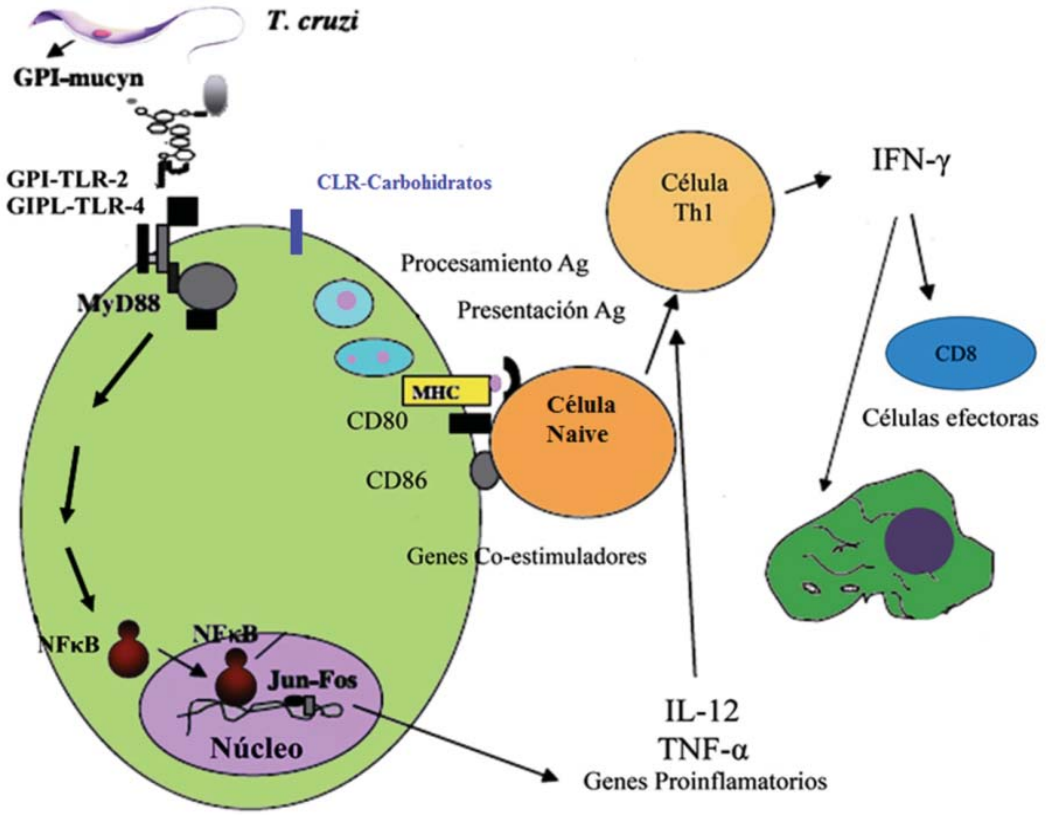


Figura 3. Respuesta Inmune contra *T. cruzi*. Las moléculas GPI y GPIL de *T. cruzi* son reconocidas por TLR-2 y TLR-4 respectivamente. Después del reconocimiento se activa la vía dependiente de MyD88 la cual culmina con la activación del factor de transcripción NF-κB la cual transloca en el sitio de genes inflamatorios para la síntesis de citosinas proinflamatorias como IL-12 y TNF-α que en conjunto con las moléculas de presentación de antígeno (MHC-II CD-80 y CD86) activan al linfocito T naive hacia un perfil Th1 el cual produce IFN-γ para la activación de linfocitos $CD8^+$ y la eliminación de células infectadas. Los receptores de tipo lectina dependientes de calcio (CLRs) pueden reconocer antígenos con carbohidratos para fagocitar y presentar péptidos de *T. cruzi.*

Receptores Tipo lectinas Dependientes de Calcio

La superfamilia de receptores tipo lectina dependientes de calcio (CLRs) son grupo de proteínas que se caracterizan por la presencia de uno o más dominios tipo lectina de tipo C (CTLD). La superfamilia se divide en 17 grupos basados en su filogenia y organización del dominio [47]. Los CLRs son un grupo de receptores que juegan papel importante en la inmunidad contra patógeno [48]. Estos receptores se encuentran presentes en la membrana en una forma soluble en el plasma y tienen un o más dominios de reconocimiento de carbohidratos (CRD) [49]. Los

CLRs reconocen e internalizar antígenos de carbohidratos específicos de manera dependiente de Ca^{2+} [50-52]; además, de estar implicados en diversos fenómenos, como: desarrollo embrionario, tráfico intracelular, reconocimiento de células, el homing celular, la endocitosis, activación del complemento, y la diseminación metastásica de las células cancerosas [53, 54]. El reconocimiento inicial de un patógeno por las células presentadoras de antígenos (CPAs) como Mφ o DCs es determínate para dirigir la respuesta de células T efectoras a una respuesta inmune [49, 53].

Los CLRs desempeñan un papel importante en la activación/maduración de CPAs al unirse con PAMPs [55], el dominio de reconocimiento de carbohidrato (CRD) reconoce los carbohidratos específicos de la superficie de patógenos [49, 53], además los CLRs solubles inducen aglutinación, inmovilización, opsonización mediada por el complemento y lisis [50]. El receptor de Adhesión específica intercelular de molécula-3-grabbing no integrina de las células dendríticas (DC-SIGN), el receptor de manosa (MR) y el receptor de MGL (del inglés Macrophage galactose- type C Lectin) es un CLRs importantes en el reconocimiento del parásito *Schistosoma mansoni* (SEAs)[56]. De estos tres CLRs mencionados, el menos estudiado es MGL.

MGL es una glicoproteína transmembranal con un dominio de cuello y CRD formada por una estructura con tres puentes disulfuro [57]. MGL se expresa selectivamente en DCs inmaduras y Mφ, en humano solo existe un tipo de receptor hMGL; mientras que en ratón tienen dos copias; mMGL1 y mMGL2 ambos contienen una secuencia Gln-Pro-Asp en su CDR que facilita el reconocimiento de galactosa (Gal) o N-acetilgalactosamina (GalNAc)[52, 58]. MGL1 es altamente específico para el reconocimiento de estructuras Lewis X (Le^x) y Lewis A (Le^A), mientras que MGL2 tiene especificidad por Gal y GalNAc. Recientemente se ha reportado en células dendríticas (DCs) la capacidad de mMGL para reconocer ligandos solubles conduciéndolos por vía endosoma-lisosoma para su degradación y presentación en moléculas MHC-II y en consecuencia favoreciendo la activación de células T "cooperadoras" (Tabla 1) [59].

La membrana celular de *T. cruzi* presenta glicoproteínas de tipo mucina, éstas están ancladas en GPI y GPIL. Se ha repostado que la purificación de estas moléculas provenientes de tripomastigotes de *T. cruzi* (tGPI-mucina) activan las células del hospedero, favoreciendo la activación de proteínas quinasas activadas por mitógenos (MAPK) y factores de transcripción relacionados en Mφ mostrando una fosforilación de ERK1/2, proteína quinasa activada por estrés (SAPK) quinasa-1 / proteína quinasa activada por mitógeno (MAPK) quinasa-4 y p38/SAPK-2, después de 15 y 30 minutos del contacto con tGPI-mucina TNF-α e IL-12 sensibilizados con IFN-γ y expuestos a tGPI [60].

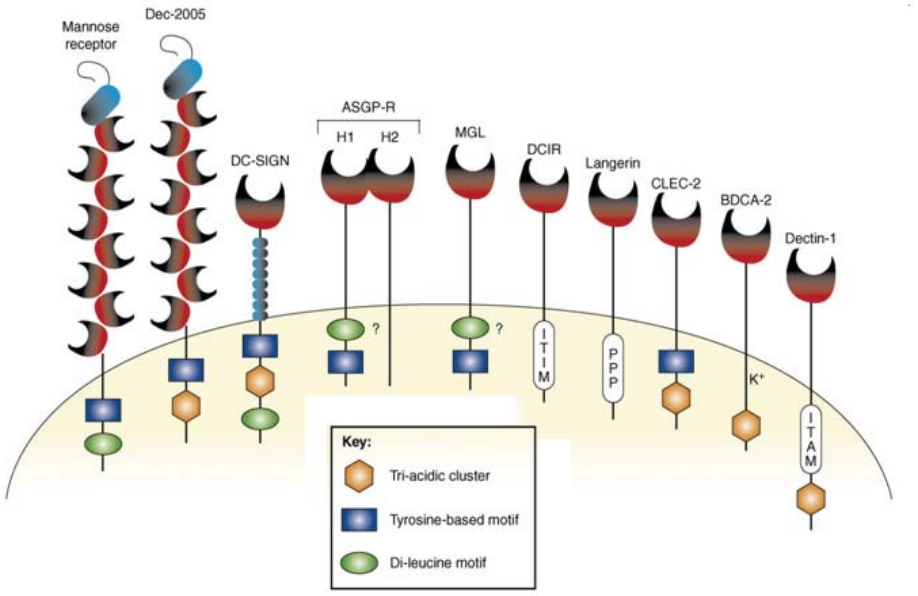


Figura 4. Motivos de internalización y señalización de CLRs. Los motivos de internalización se encuentran en los dominios citoplásmicos de la lectina de tipo C y moléculas de tipo lectina tipo C. Estos motivos incluyen el grupo triacídico, un motivo de internalización basado en tirosina, y un motivo de di-leucina. Solamente un motivo parcial de di-leucina está presente dentro de la región citoplasmática de MGL y ASGP-R H1. Las lectinas de tipo C DCIR y Dectin-1 contienen un ITIM clásico (inhibidor) y un ITAM parcial (activación), respectivamente. Langerin posee un estiramiento rico en prolina que podría servir como un punto de acoplamiento para las proteínas que contienen el dominio SH3. El residuo de lisina cargado positivamente en BDCA-2 facilita la asociación de BDCA-2 con la cadena común de FcR g. Para mayor claridad, los receptores se muestran como moléculas individuales, mientras que en la membrana celular estos receptores pueden expresarse como oligómeros. ASGP - R, receptor de asialoglicoproteína; BDCA - 2, antígeno de células dendríticas de sangre 2; CLEC, receptor tipo 2 similar a lectina de tipo C; DCIR, gen inmunorreceptor de células dendríticas; DC-SIGN, nonintegrin aglutinante intercelular específico de células dendríticas; DEC-205, receptor de células dendríticas y epiteliales de 205 kDa; la lectina de galactosa de macrófago (MGL), (Imagen tomada de Van Vliet S.J. *et al.* 2008 [61]).

Sin embargo, los mecanismos o vías que utiliza mMGL, para la internalización de los carbohidratos no han sido completamente descritos. El receptor de tipo lectina conocido como ASGP-R el cual contiene motivos intracelulares idénticos a mMGL y estos son fosforilados en los residuos de serina y tirosina regulando así la activación del receptor, esto abre la posibilidad de que mMGL pueda señalizar [59]. Algunos estudios sugieren que el receptor MGL se ve implicado en la presentación de antígenos tanto en los Mφ como en las CDs, además de influir en la producción de citocinas que modulan la respuesta inmune innata [62, 63].

Tabla 1. MGL ortólogos en humano, ratón y rata.

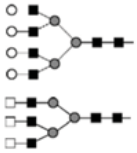
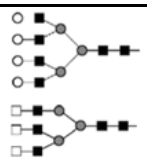
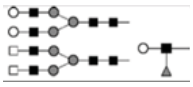
Especies	Ortólogos nomenglatura	Chromosomal localización	Patrón de Expresión	Especificidad de carbohidratos	Estructura de glicanos reconocidos
Humano	**ASGP-R(H1/H2)**	17p13.2	Hepatocitos	GlicanosTri-o tetra-antenario con terminal	
Humano	**MGL** (CD301 corto) **DC-ASGPR** (CD301 largo)	17p13.2 17p13.2	CDs inmaduras Mφ CDs inmaduras Mφ	Terminal GalNAc (Tn antigen, LDN) No determinado	
Ratón	**mMGL1 (MGL1)**	11 B3	CDs inmaduras Mφ	Lewis X Galactosa terminal GalNAc teminal	
Ratón	**mMGL2 (MGL2)**	11 B3	CDs inmaduras Mφ	GalNAc teminal	
Rata	**rMGL** (M-ASGP-BP)	10q24	Mφ y CDs	Bi-antenanay glycans con gal/GalNac, Lexis X, Lewis A terminal	

Tabla 1. Ligandos de estructuras glicanas representativas para MGL de humano, ratón y rata. Estructura teórica de un tria-tenaria de epítopes que contienen N-glycan terminal GalNAc. LDN, LacdiNac; □ , GalNAc; ○, galactosa; ■, GlcNAc; •, manosa; ▲, fucosa (tabla tomada de [61]).

Trabajos previos realizados en nuestro laboratorio [64], han demostraron que el parásito *T. cruzi* (cepa Querétaro) tiene antígenos glicosilados que pueden ser reconocidos por el receptor MGL (específicamente Gal-NAc). Cuando ratones deficientes para el gen *mgl1* (mMGL$^{-/-}$) fueron infectados con *T. cruzi* se observó un incremento significativo en la mortalidad y esto se asoció con una mayor parasitemia en sangre, así como un mayor número de nidos de amastigotes en corazón en comparación con ratones silvestres (WT). Además, Mφ peritoneales con 21 días post infección de ratones MGL1$^{-/-}$ presentaron una producción disminuida de óxido nítrico (NO), IL-12, TNF-α e IL-10 en comparación con los WT [64]. Estas evidencias apuntan a que MGL1 puede tener un papel importante en la activación de los Mφ, células del sistema inmunitario innato, siendo críticas para el control inicial de la infección por *T. cruzi*.

Sin embargo, aún se desconocen los detalles de la participación de MGL1 en la activación de los Mφ. En este trabajo se aborda el estudio de MGL1 en Mφ infectados *in vitro* con *T. cruzi* con el fin de entender el o los mecanismos de MGL1 en la activación de los Mφ en la infección. Utilizamos Mφ de ratones MGL1$^{-/-}$ y WT que se infectaron *in vitro* con epimastigotes de *T. cruzi*, corroboramos *in vitro* las observaciones previas hechas en la infección *in vivo*: los Mφ de ratones MGL1$^{-/-}$ infectados *in vitro* se infectan más. Comprobamos que los Mφ MGL1$^{-/-}$ expresan significativamente menos TLR-2 y TLR-4 producen menos TNF-α e IL-12 en respuesta al LPS ó al antígeno de *T. cruzi* (Ag*Tc*) y en co-cultivos de Mφ MGL1$^{-/-}$ esplenocitos se observó una deficiente producción de TNF-α, IL-10, IFN-γ, IL-4 e IL-13. Estas observaciones sugieren que mMGL juega un papel crítico en la resistencia de *T. cruzi*. Así mismo, cuando Mφ WT y ASC$^{-/-}$ se trataron con inhibidores de caspasa-1, IL-1β o NADPH oxidasa, se descubrió que la producción de IL-1β por caspasa-1/ASC del inflamasoma requería especies reactivas de oxígeno (ROS) como señal secundaria. Además, de que la IL-1β reguló la señalización de NF-kB. Mφ NLRP3$^{-/-}$, a pesar de la incapacidad de provocar la activación de IL-1b y la expresión del gen de

la citocina inflamatoria, mostraron una disminución de 4 veces en los parásitos intracelulares en comparación con lo observado en los controles WT coincidentes [65].

Justificación

El receptor de galactosa tipo lectina C de macrófago (MGL) es un receptor expresado en macrófagos (Mφ) y células dendríticas (CDs). MGL1 reconoce de manera específica residuos de galactosa teminal (Gal) y N-Acetilgalactosa terminal (GalNAc) expresados en la superficie de *T. cruzi*. Sin embargo, se desconoce el papel inmune que desempeña MGL en la función y activación de los macrófagos (Mφ) en la infección por *T. cruzi*.

Estos resultados nos permitirán:

1) Establecer la participación de MGL1 en la activación de los Mφ.

2) Establecer la participación de MGL1 en la producción de citocinas que se genera en la infección por *T. cruzi*.

3) Establecer posibles vías de señalización que son activadas por MGL1 en el Mφ.

Hipótesis

El receptor MGL1 participa de en la activación, fagocitosis, lisis y presentación antigénica de los Mφ para controlar la infección *in vitro* por *T. cruzi*

Metas

1. Establecer si MGL1 es importante en la internalización y eliminación *T. cruzi* en Mφ infectados *in vitro.*
2. Establecer la participación de MGL1 en la activación de los Mφ infectados *in vitro* con *T. cruzi* a través de la determinación de la expresión de TLR-2, TLR-4, CD40, CD80 y MHC-II.
3. Establecer la participación de MGL1 en la producción de TNF-α e IL-12 por Mφ en la infección *in vitro* por *T. cruzi.*
4. Establecer la participación de MGL1 en la activación (proliferación celular) de esplenocitos en la infección por *T. cruzi*.
5. Establecer la participación de MGL1 en el tipo citocinas producidas por los linfocitos T en la infección con *T. cruzi*.
6. Establecer las posibles vías de señalización de MGL1 que participan en la activación de los Mφ infectados *in vitro* con *T. cruzi.*
7. Establecer la participación de MGL1 en la síntesis del NLPR3 así como la producción de IL-1β.

Objetivos

Objetivo General

- Establecer la participación de MGL1 en la activación y funcionalidad de los Mφ en la infección *in vitro* por *T. cruzi.*

Objetivos Particulares

- Determinar el porcentaje de Mφ peritoneales WT y MGL1$^{-/-}$ infectados *in vitro* con *T. cruzi.*
- Determinar la capacidad trypanocida de Mφ WT y MGL1$^{-/-}$ en la infección *in vitro* con *T. cruzi*.
- Determinar la producción de óxido nítrico (NO), TNF-α, IL-12 e IL-10 en el sobrenadante de los Mφ MGL1$^{-/-}$ y WT infectados *in vitro* con *T. cruzi.*
- Analizar la participación de MGL1 en la activación de los Mφ infectados *in vitro* con *T. cruzi* a través de la determinación de moléculas co-estimuladoras (CD40, CD80, y MHC-II), y de los receptores TLR-2 y TLR-4.
- Determinar, en un modelo *in vitro* de co-cultivo Mφ-Linfocitos T, la funcionalidad de los Mφ MGL1$^{-/-}$ y WT para la activación linfocitos T de memoria provenientes de la infección con *T. cruzi*, por incorporación de ^{3}HTdR.
- Determinar, en un modelo *in vitro* de co-cultivo Mφ -Linfocitos T, los niveles de citocinas IL-12, IFN-γ, IL-4, TNF-α e IL-10).
- Determinar las posibles vías de señalización activas en Mφ WT y MGL$^{-/-}$ estimulados *in vitro* con Ag total de *T. cruzi*

Metodología

Ratones. Se utilizaron ratones de 6-9 semanas de edad WT y ratones MGL$^{-/-}$ un fondo genético C57BL/6 (Donados por Glycomics Consortium, USA). Los ratones han estado en reproducción por más de 10 generaciones en el bioterio de la FES-Iztacala. Los ratones WT con fondo genético C57BL/6 se adquirieron de Harlan (México) y se utilizaron como controles. Todos los estudios con animales se apegaron a los lineamientos para el cuidado y uso de animales de laboratorio, de la norma Mexicana NOM-062-ZOO-1999, 2001.

Confirmación de la presencia o ausencia de la molécula de MGL en ratones WT y MGL$^{-/-}$ por PCR. A fin de confirmar que los ratones cumplan con la condición silvestre WT o MGL$^{-/-}$, se obtuvo DNA del apice de la cola (<0.5 cm) de cada ratón por la técnica convencional de lisis con proteinasa K (Invitrogen) y se amplificó el gen para *mmgl* (mgl de raton) y *neomicina* por la técnica de PCR. Los primers y condiciones usadas fueron las siguientes: mMGL-F (5′-CTTGGTCCCAGATCCGTATC), mMGL-R (5′-ATGTCATGACTCAGGATC), NEO-F (5′-AGGATCTCCTGTCATCTCACCTTGCTCCTG) y NEO-R (5′-AAGAACTCGTCAAGAAGGCGATAGAAGGCG) (Todos sintetizados por Sigma-Aldrich, México). con la ADN polimerasa (Amplificasa, marca BioTecMoI) 0.5 µl, y DNTP's (Invitrogen, 0.2mM) se utilizó la temperatura de desnaturalización 94°C, de alineación 55° C para NEO y mMGL respectivamente, y de elongación 72°C, por 35 de ciclos.

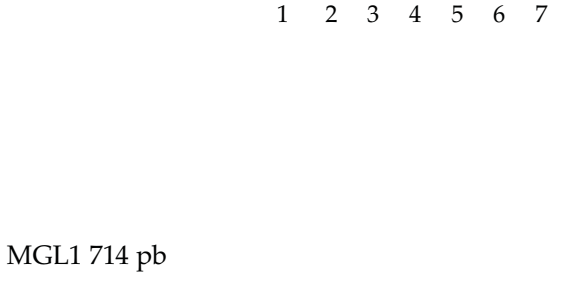


Figura 5. Genotipo de ratones wild type y MGL1$^{-/-}$. Corrimiento electroforético que demuestra el genotipo de cada uno de los ratones. En los carriles 2 y 3 podemos observar la presencia del gen *mmgl* (633pb) en ratones WT, los carriles 4 y 5 muestran el gen de inserción de *neomicina* (492pb) en ratones MGL$^{-/-}$. En el carril 6 se muestra el control, es decir únicamente los componentes de la reacción de PCR sin DNA con la finalidad de mostrar que los reactivos no se encuentran contaminados, el carril 7 contiene DNA sin amplificar con la finalidad de mostrar que no se encuentra degradado.

Parásitos. En este trabajo se utilizó la cepa mexicana *T. cruzi* TBAR / MX / 0000 / Querétaro perteneciente a DTU TcI. Se cultivaron epimastigotes de *T. cruzi* a 28 °C en cultivo bifásico con caldo de infusión de cerebro y cerebro, agar y dextrosa (Sigma-Aldrich, CDMX, MX), y en la fase líquida con solución salina suplementada con 5% de bovino fetal inactivado por calor. suero (FBS, Thermo Fisher Scientific, MA, EE. UU.) con 100 U de penicilina / estreptomicina (todas de GIBCO-BRL, NY, EE. UU.).

Antígeno soluble de lisado de *T. cruzi* (Ag*Tc*). Se obtuvieron epimastigotes derivados de cultivos y se lavaron tres veces en solución salina tamponada con fosfato (PBS) mediante centrifugación a 1.300 xg durante 10 min. El sedimento obtenido se sonicó seis veces durante 10 segundos cada uno a 50 W usando un Dimem-brator sónico 300 (Thermo Fisher Scientific) en presencia de inhibidores de la proteasa (Sigma-Aldrich). La lisis de los parásitos se confirmó utilizando un microscopio. Los lisados de parásitos se centrifugaron a 20.000 xg durante 30 minutos a 4° C, y los antígenos solubles en PBS se almacenaron a -70° C hasta su uso. La concentración de proteína se determinó utilizando un kit de proteína Bradford (Sigma-Aldrich).

Determinación de la expresión de MGL1 y MGL2 en Mφ MGL1$^{-/-}$ y Mφ WT [44, 66] . Se obtuvieron células totales de la cavidad peritoneal (PECs) de ratones sanos WT y MGL1$^{-/-}$ por el método de exudado peritoneal, la purificación de Mφ se realizó por el método de adherencia en placa. Los

Mφ obtenidos fueron ajustados a 10^6 células/ml en medio DMEM suplementado con 5% de suero fetal bovino (FCS) 1% de penicilina-streptomicina (*In vitro*) y sembrados en placas de 12 pozos. Los Mφ se estimularon con antígeno total de *T. cruzi* (Ag*Tc*) cepa Queretaro por 24 ó 48h. Posteriormente, los Mφ fueron colectados y marcados para su análisis por citometría de flujo. Brevemente, los Mφ fueron incubados con el anticuerpo FcγR anti-ratón (CD16/CD32), para evitar el pegado inespecífico de los anticuerpos de interés. Para identificar la población de Mφ se utilizó el anticuerpo monoclonal específico para ratón F4/80 conjugado con FITC, para MGL1 conjugado con PE y para mMGL-2 se utilizó un anticuerpo que tiene especificidad tanto para mMGL1 como para MGL2 conjugado con APC, esto debido a que no existe en el mercado un anticuerpo específico solo para mMGL2 (todos los anticuerpos de marca Biolegen). La determinación de su expresión se realizó en un citómetro BD FACS Calibur.

Cultivo de Mφ peritoneales e infección *in vitro* con *T. cruzi*. Se obtuvieron PECs de ratones WT y MGL1$^{-/-}$ por el método descrito en la parte superior. Los Mφ obtenidos se ajustaron a 10^6 células/ml en medio DMEM suplementado con 5% de suero fetal bovino (SFB) 1% penicilina-streptomicina, se sembraron en placas de 12 pozos que contenían en el fondo del pozo cubreobjetos circulares estériles. Una vez sembrados, se incubaron por 2h para permitir su adherencia, se lavaron para descartar las células no adheridas. Las células adheridas fueron infectadas con *T. cruzi* cepa Queretaro; *T. cruzi* previamente cultivados *in vitro*, relación 1:10 (un Mφ por cada diez parásitos). Los Mφ se cultivaron durante 48 hr a 37° C en una estufa de CO^2 a 5% humedad [64].

Tinción de Mφ infectados *in vitro*. Para determinar el porcentaje de Mφ infectados, así como el número de parásitos internalizados por célula. Se colocaron portaobjetos circulares dentro de placas de 12 pozos; Posteriormente se depuraron Mφ por el método de adherencia y se estimularon por 24h con LPS (0.2μg/ml), IFN-γ (1μg/ml) Ag*Tc* (25μg/ml) o sin estimulo (únicamente medio cultivo). Se infectaron los Mφ con *T. cruzi* por 2 hr. Se recuperaron los

cubreobjetos con y se fijaron con alcohol metílico, se teñieron con colorante GIEMSA (Sigma-Aldrich), siguiendo las especificaciones del fabricante, para su posterior observación al microscopio de luz (Zeiss Ax10 Vert A1). Se contó el número de parásitos internos en 100 células para cada grupo experimental, por triplicado, a doble ciego. Se analizaron dos experimentos independientes.

Ensayo trypanocida por incorporación de timidina tritiada (^{3}H-TdR). Se obtuvieron Mφ WT y MGL1$^{-/-}$ de ratones sanos, por el método de adherencia previamente descrito. Las se ajustaron a 10^6 células/ml y se sembraron en placas de 12 pozos y fueron estimuladas por 24h con estímulo de LPS (0.2µg/ml), Ag*Tc*, 25µg/ml o sin estimulo (únicamente medio). Se infectaron relación 1:10 con parásitos de *T. cruzi*, transcurridas 2 hrs de infección las células se lavaron 3 veces con DMEM para eliminar los parásitos no internalizados. Las células se incubaron por 24 hrs, se lisaron con 0.01% de duodecil sulfato de sodio (SDS) en medio de DMEM no complementado por 10 minutos. Los amastigotes liberados se resuspendieron en 600µl de medio DMEM complementado y se sembraron por cuadruplicado (150µl/pozo) en placas de 96 pozos y cultivados por 72 horas a 37°C con 5% CO^2. Para determinar la viabilidad y proliferación de los parásitos se agregó 0.5µCi/ml de 3H-TdR en 20µl de DMEM complementado por pozo 18 horas antes de cumplir las 72h. La placa fue cosechada en papel filtro fibra de vidrio (Perkin Elmer), y se cuantificaron las cuentas por minuto (CPM) empleando un contador de centelleo (Wallac Trilux 1450 microbeta).

Determinación de Óxido nítrico (NO) en sobrenadante. Se determino el NO del sobrenadante de los cultivos de Mφ por el método nitrato-reductasa (Greiss) (ThermoFisher scientific G7921). La Solución A, 0.1% de naphthylethylenediamine dihydrocloride en agua destilada; y la Solución B sulphanilamida 5% en H3PO4 al 5%). Se mezclaron la solución A y B a igual volumen. En placa para ELISA de fondo plano se colocará una curva de dilución doble (empezando en 100 mM) de nitrito de sodio (NaNO2). Tanto la curva como las muestras se colocarán en un

volumen de 100 µl y 100 µl de reactivo de Greiss. Se incubó a temperatura ambiente por 10 min y se leyó a 550-620nm en un lector de ELISA (Metertech S960).

Determinación de radicales ROS en la infección con *T. cruzi*. Se utilizaron Mφ peritoneales sembrados en cubreobjetos de se mencionó anteriormente. Se usaron Mφ en estado basal, infectados con *T. cruzi* (proporción de 1:10), estimulados con Ag*Tc* (2.5µg/900µl) y como control a Mφ infectadosn con *Candida albicans* (*C. albicans*) 1x50^6 levaduras/900µl. Previo a la infección de Mφ, las levaduras de *C. albicans* se resuspendieron en medio 900µl DMEM con SFB y se incubaron a 37° C por 1 hr. Para observar las ROS producidas por Mφ, se adicionaron 100 µl de Tetrazolio nitro azul NBT al 0.1% para llevar los pozos a un volumen final de 1 ml. Colocar 1 ml de la preparación de antígeno en cada cubreobjetos incubar a 37°C por una hr. Lavar con solución salina y retirar el exceso dejar secar los cubreobjetos con papel absorbente. Los Mφ se tiñeron con safranina al 0.5% y se dejaron por 7 min, se lavaron agua destilada. Se retiraron los cubre objetos de la placa de 12 pozos para secarse sobre papel absorbente. Se montaron los cubreobjetos en los portaobjetos con resina [67].

Determinación de citocinas de Mφ estimulados *in vitro*. Se determinó la producción de las citocinas IL-12, TNF-α, IFN-γ, e IL-10 por el método de ELISA-sandwich en el sobrenadante de los cultivos de los Mφ con los diferentes estímulos descritos en la parte superior, de acuerdo a las especificaciones del proveedor (Peprotech-México).

Determinación de viabilidad de Mφ infectados con *T. cruzi*. Se obtuvieron Mφ de ratones WT y MGL1$^{-/-}$, se ajustaron a 1x10^6 de Mφ/ml y se añadieron 100µl de estos por pozos en una placa de cultivo de 96 pozos, usándose 4 pozos por tratamiento, después de 24hr de incubación a 37°C. Los parásitos de *T. cruz*i se ajustaron a 1x10^6 por 200µL en DMEM suplementado por 2hr, después se lavaron los pozos para eliminar los parásitos no internalizados. Posteriormente, a todos los pozos se les agregó 25µL (5mg/ml) de MTT (3-[4,5 dimetilazol-2 y 1]-2,5 difeniltetrasolio bromuro) y se incubaron por un tiempo de cuatro horas a una temperatura de

37°C. Se centrifugó la placa a 2500 rpm durante cinco minutos y se retiró el sobrenadante, quedándose únicamente con la pastilla, a la cual se le agregó posteriormente 100µL de dimetil sulfóxido (DMSO) y se dejó 15 min en obscuridad (se utilizaron como blanco tres pozos de la placa que no contenían células a los que se le agregaron 100 µL de DMSO). Se leyó la absorbancia a 492nm.

Expresión de moléculas de Mφ *in vitro*. Se obtuvieron exudados de células totales de la cavidad peritoneal (PECs) de ratones WT y MGL1$^{-/-}$ sin infección; se ajustaron a 10^6, purificadas por el método de adherencia y cultivados en placas de 24 pozos por 24h con estímulo de LPS (0.2µg/ml), Ag*Tc* (25µg/ml) *T. cruzi* (10 parásitos/ Mφ) o sin estimulo (únicamente medio). Los Mφ fueron recuperados y se analizó la expresión de moléculas co-estimuladoras por citometría de flujo. Brevemente, los Mφ se incubaron con el anticuerpo FcγR anti-ratón (CD16/CD32), para evitar el pegado inespecífico. Para identificar la población de Mφ se utilizó el anticuerpo monoclonal específico para ratón Pacific blue F4/80, PerCP/Cy5.5-CD11b, PE-TLR-4, PE-MHC-II, FITC-TLR-2, FITC-CD40 y FITC-CD80 (Todos marca BioLegend; San Diego, CA, USA.). Se siguió el procedimiento sugerido por el proveedor y se cuantificaron las muestras en el equipo BD FACS Calibur.

Obtención de esplenocitos. Para los co-cultivos se utilizaron ratones WT infectados con *T. cruzi* 21 días post-infección (dpi), se extrajeron los esplenocitos del bazo por perfusión utilizando solución salina. Las células se contaron y se ajustaron a $1x10^7$ cel/ml.

Co-cultivos de Mφ-esplenocitos. Se obtuvieron Mφ MGL1$^{-/-}$ y WT provenientes de ratones sanos por el método de adherencia descrito en la parte superior. Se contaron y ajustaron a $1x10^6$ cel/ml. Los Mφ se sembraron en placas de 24 pozos, incubaron por 2 h para permitir su adherencia, se lavaron para retirar las células no adheridas y se agregó Ag*Tc* (25 µg/ml) y se incubaron por 2 h, nuevamente se lavaron al menos 3 veces para retirar el antígeno no fagocitado. Se agregaron

los esplenocitos de ratones WT infectados en proporción 1:10 (Mφ-esplenocitos). Se cultivaron por 72 h.

Proliferación celular. De los co-cultivos (esplecnocitos-Mφ) se añadió (3-HTdR) (185 GBb/mmolactivity, Amersham England) 1 μCi/pozo. Las células fueron cosechadas y se contabilizara por incorporación de (^{3}H)-TdR después de 72 h de estímulo.

Determinación de citocinas de esplenocitos en co-cultivo con Mφ. En el sobrenadante de los co-cultivos Mφ-esplenocitos se determinó IL-4, IL-13, IL-10, IFN-γ, IL-12. Se utilizó el método de ELISA sándwich de acuerdo a las especificaciones del proveedor (Peprotech).

Niveles de proteínas de las vías de señalización P38, ERK1/2, c-jun, NFκB y NLRP3 detectadas por transferencia de Western. Los Mφ derivados de la médula ósea murinos (BMMφ) se generaron utilizando tibias y fémures extraídos asépticamente de ratones MGL1$^{-/-}$ y WT como se describió anteriormente [24]. En resumen, los extremos de los huesos se cortaron y se lavaron con 10 ml de PBS estéril. La suspensión celular obtenida se centrifugó a 1.300 xg durante 10 minutos a 4ºC. Las células se ajustaron a una concentración de 4 x 10^6 células/ml en medio de diferenciación de Mφ que contenía DMEM suplementado (FBS al 20%) y 50 ng/ml de factor estimulante de colonias de Mφ murinos (M-CSF) (Biotech, BG, DE). Se sembraron dos mililitros de suspensión celular en cada pocillo de una placa de 6 pocillos y se incubaron a 37 ° C en 5% de CO^2. Después de 72 h, se añadió 1 ml de medio de diferenciación a cada pocillo. Las células se dejaron diferenciar durante 7 días. Los BMMφ se lavaron dos veces, se ajustaron a una concentración de 4 x 10^6 células / ml y se estimularon con LPS (100 ng / ml) o Ag*Tc* (25 μg / ml) durante 0, 5, 15 y 30 minutos. La proteína BMMφ se extrajo utilizando un tampón Laemmli (que contiene Tris 92 mM (pH 6,8), glicerol al 18%, SDS al 1,8%, azul de bromofenol al 0,02% y β-mercaptoetanol al 2% (todos de Sigma-Aldrich) con inhibidores de proteasa y fosfatasa (Roche Diagnostic, Basel Suiza) de acuerdo con las instrucciones del fabricante. Las muestras se centrifugaron a 700 xg durante 5 min a 4 ° C y la concentración de proteína se determinó utilizando un ensayo de

Bradford (Sigma-Aldrich). Las muestras de proteína (15 μg) separados por electroforesis en gel de dodecilsulfato de sodio y poliacrilamida (SDS-PAGE) al 12% a 80 V y se transfirieron a membranas de inmobilon-P (0,22 μM, Millipore, MA, EE. UU.) mediante electrotransferencia. Las membranas se bloquearon durante 2 ha temperatura ambiente. en solución salina tamponada con Twis-Tween 20 (TBST) suplementada con 5% p / v de albúmina de suero bovino (Sigma-Aldrich). Posteriormente, la membrana se lavó tres veces con TBST y se incubó a 4 ° C durante la noche en una placa agitadora con siguientes anticuerpos primarios: GAPDH (como housekeeping protein), NFκB p65, p-NFκB p65, p38 MAPK, p-p38 MAPK, p44/42 MAPK (Erk1,2), p-p44 / 42 MAPK (p-Erk1,2) y NLRP3 siguiendo las indicaciones del fabricante protocolo (Cell Signaling, MA, USA). Después de lavar la membrana con TBST cuatro veces, se añadió un anticuerpo secundario conjugado con fosfatasa alcalina en TBST (dilución 1: 5000; Señalización celular) y se incubó durante 2 horas a temperatura ambiente. La membrana se lavó con TBST cuatro veces, la señal se reveló utilizando Super Signal West Femto (Thermo Fisher Scientific) y luego se escanearon y analizaron con un escáner infrarrojo fluorescente Odyssey (LI-COR Lincoln, NE, EE. UU.).

Análisis estadístico. Se utilizó el programa GraphPath Prism 6. Se aplicó la prueba de ”t” de Student, long rang test y Wilcoxon según correspondiera a cada caso.

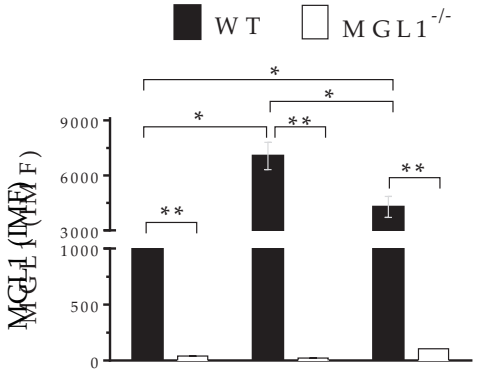
Resultados

Los Mφ de ratones MGL$^{-/-}$ tiene una supresión del receptor MGL1. Los ratones tienen dos genes de MGL, denominados MGL1 y MGL2, con distintas funciones y distribuciones. Se ha reportado que existen niveles muy bajos de MGL2 en Mφ peritoneales, mientras que MGL1 tiene una mayor expresión. Mφ peritoneales y diferenciados de medula ósea (BMMφ) se expresa mayormente MGL1 que MGL2 [68]. Ya que cuando se realizaron estos ratones suprimidos para el receptor mMGL no se había reportado la presencia de MGL2[66, 69]; nosotros medimos por citometría de flujo la intensidad media de fluorescencia (IMF) de los anticuerpos específicos para MGL1 y MGL2 en Mφ (F4/80^{+}) estimulados *in vitro* por 24 ó 48 h con antígeno de *T. cruzi* (Ag*Tc*) para determinar la supresión de MGL1, MGL2 o ambos. Como se esperaba, los ratones WT tuvieron una expresión del receptor MGL1 y cuando se estimulan con Ag*Tc* esta expresiónaumenta, tanto en Mφ residentes del peritoneo como diferenciados de medula ósea. Los Mφ MGL$^{-/-}$ muestran una disminución en la expresión de MGL1 tanto sin estimulo como con estimulo de Ag*Tc* en ambos tipos de Mφ. Además, se observó la presencia de MGL2 en ratones MGL$^{-/-}$ en estado basal en niveles iguales a los ratones WT (Fig 6. A y B). Con estos resultados concluimos que los ratones MGL$^{-/-}$ son deficientes para MGL1, por los que a partir de este resultado los mencionaremos como MGL1$^{-/-}$.

a) Mφ residentes del peritoneo

Sin-estímulo 24h 48h

MGL1 (expresión)



b)

MGL2 (expresión)

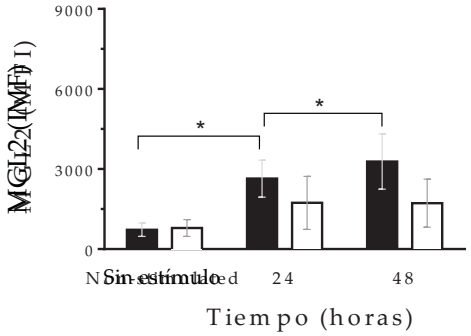


Figura 6. Expresión de MGL1 y MGL2 en Mφ de ratones WT y MGL1$^{-/-}$. (**a-b**) histograma de la expresión de MGL1 y MGL2 en Mφ infectados *in vitro* con *T. cruzi* , línea punteada isotipo; linea negra Mφ de ratones MGL1$^{-/-}$; área gris Mφ de ratones Wild-Type (WT). Gráfico de barras de intensidad media de florecencia de Mφ que expresan MGL1 y MGL2. n=6 ratones por grupo. *=p<0.05.

Los Mφ MGL1$^{-/-}$ tiene un mayor porcentaje de amastigotes de *T. cruzi*. mMGL ha sido descrito como un receptor involucrado en procesos de fagocitosis. Nosotros evaluamos la participación de MGL1 en la internalización de *T. cruzi*. Se obtuvieron Mφ peritoneales de ratones WT y MGL1$^{-/-}$, y se infectaron *in vitro* con *T. cruzi*. Nuestros resultados muestran que los Mφ MGL1$^{-/-}$ tiene un mayor porcentaje de Mφ infectados, con un total de los con un 86% infectados ; siendo del 100% de los Mφ MGL1$^{-/-}$ un 33% infectados con 2 o 3 parásitos. En contraste, los Mφ WT tuvieron un 48% de los Mφ infectados, en donde se observó de un 100% de los Mφ WT el 29% de los Mφ WT con 1 parásito, (fig. 7 8 a-d). Este resultado fue congruente con la observación previas realizadas por Vázquez y colaboradores en donde se menciona que los ratones MGL1$^{-/-}$ infectados con *T. cruzi in vivo* desarrollaban mayor parasitemia en sangre a partir de los 21 días post infección, y sucumbían en mayor número que los ratones WT [64].

		Número de amastigotes por Macrófago				
	Mφ infectados/ 100 células	1	2-3	4	5	≥6
WT	48%	29%	14%	4%	1%	0%
MGL1$^{-/-}$	86%*	22%*	33%*	21%*	7%*	3%

Figura 7. Numero de parásitos internalizados. Mφ de ratones WT o MGL1$^{-/-}$ infectados *in vitro* con *T. cruzi* (proporción 1:10) por 2hr. 500 células contadas por grupo. Células totales de 2 experimentos independientes.

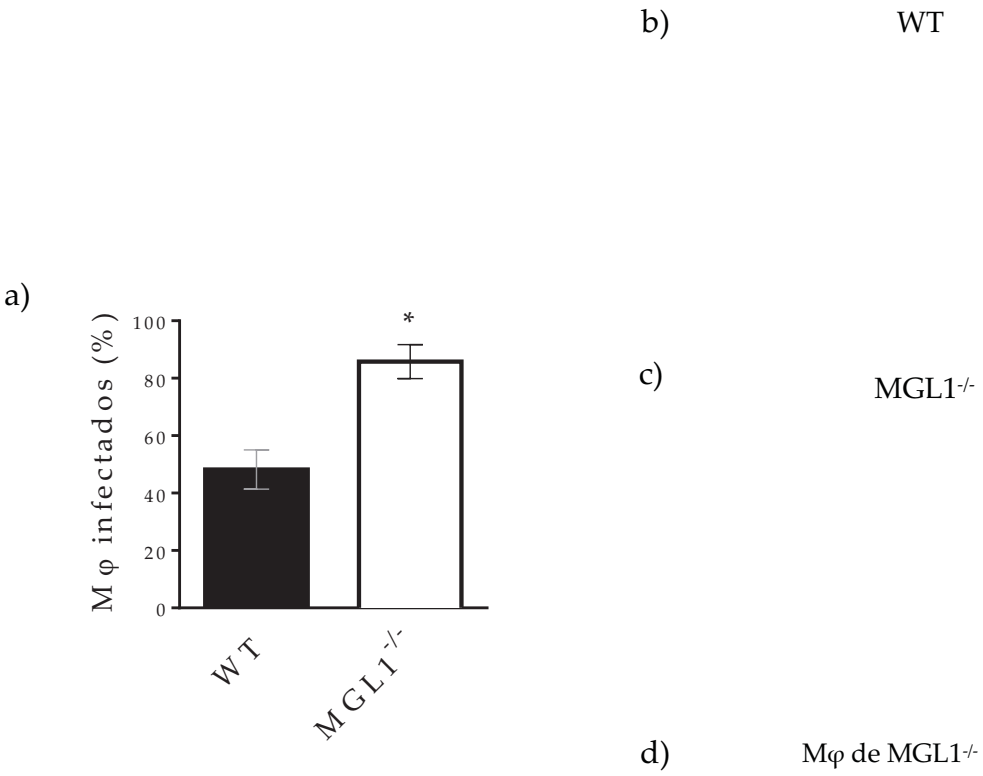


Figura 8. Infección de Mφ con *T. cruzi* . A-C) Fotografía en microscopio a 40X, Mφ WT y MGL1$^{-/-}$ infectados con *T. cruzi* . D) Porcentaje de Mφ con parásitos internos. Datos obtenidos de 2 experimentos independientes. Relación (1:10; Mφ-parásitos) ***=p<0.0006.

Los Mφ MGL1$^{-/-}$ tienen disminución de activación en respuesta a IFN-γ y LPS. Debido a que los Mφ MGL1$^{-/-}$ presentaron un mayor número de parásitos internos, nos cuestionamos si los procesos para la eliminación de *T. cruzi* se encontraban disminuidos en Mφ MGL1$^{-/-}$. Estudios previos han demostrado que la activación de los Mφ por IFN-γ es uno de los mecanismos más importantes en el control y eliminación de los parásitos *T. cruzi* [70, 71]. Así mismo, se ha reportado que un doble estímulo en Mφ con IFN-γ y LPS producen una mayor cantidad NO correlacionando con mayor eliminación de *L. major* y *T. cruzi* internos, en comparación con los Mφ insensible a Interferón Gamma (miig) [72]. Nosotros usamos Mφ WT y MGL1$^{-/-}$ y/ó estimulados con IFN-γ (1μg/ml) o LPS+IFN-γ (100 ng/ml de LPS) o sin estímulo (Basal) para observar el papel de MGL en la activación Mφ por 24 h. Los Mφ se infectaron con *T. cruzi* marcado con CSFE por 2 h. Nuestros resultados muestran un decremento en la cantidad parásitos internalizados en Mφ WT y Mφ MGL1$^{-/-}$ cuando son estimulados con LPS o IFN-γ. Sin embargo, se observan una mayor cantidad de parásitos internos en Mφ MGL1$^{-/-}$ después de la activación con LPS o IFN-γ mostrándose diferencias significativas con los Mφ WT (Fig. 9a, b). Estos resultados no dejan claro si las diferencias entre Mφ WT y Mφ MGL1$^{-/-}$ después de la estimulación con IFN-γ o LPS+IFN-γ se debe a que los Mφ MGL1$^{-/-}$ internalizan una mayor cantidad de parásitos. Sin embargo, estos resultados muestran que la ausencia de MGL1 no evita la activación del Mφ. Estos resultados concluyen que MGL1 es una molécula importante para el control de la infección por *T. cruzi*, y tiene participación en el proceso de activación de los Mφ.

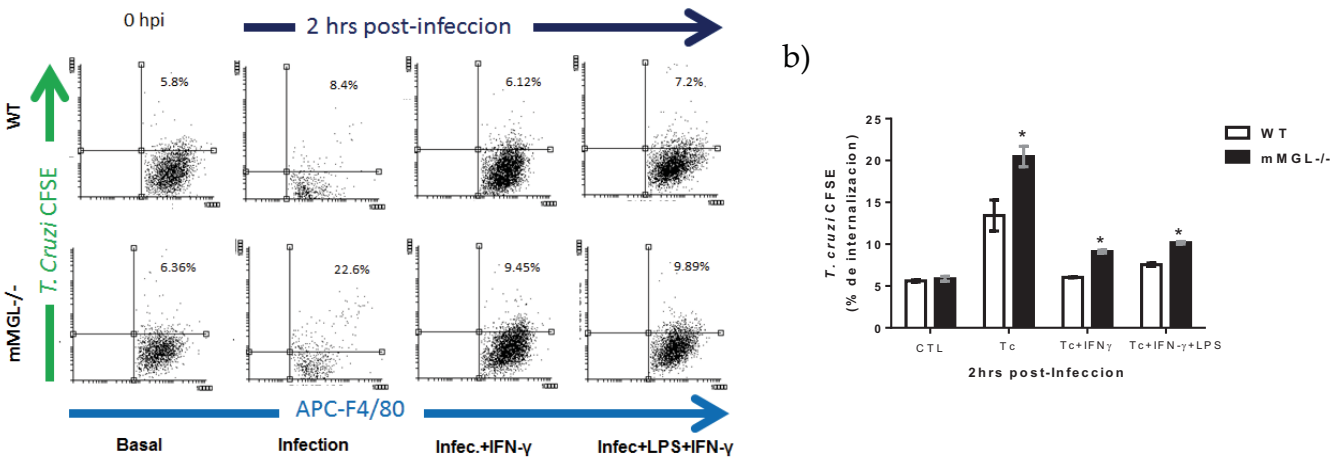


Figura 9. Infección de Mφ estimulados con LPS o IFN-γ e infectados con *T. cruzi*. Mφ WT y MGL1$^{-/-}$ fueron infectados *in vitro* con *T. cruzi* a una proporción de 1:10 (Mφ/parásitos). A) citometrías de Mφ WT y MGL1$^{-/-}$, IFN-γ (1µg/ml), LPS (0.2µg/ml). B) porcentaje de Mφ con parásitos internos. Los gráficos son representativos de dos experimentos independientes* p<0.05.

Los Mφ MGL1$^{-/-}$ tienen disminuida la capacidad de eliminar a *T. cruzi*. Ya que observamos que los Mφ MGL1$^{-/-}$ tenían un mayor número de parásitos internos después de ser estimulados con IFN-γ, ó IFN-γ+LPS en comparación con Mφ WT, ahora quisimos saber la viabilidad de estos parásitos internos. Se realizaron cultivos de Mφ MGL1$^{-/-}$ y WT sin estímulo y con IFN-γ ó LPS+IFN-γ por 24 h y posteriormente infectados con *T. cruzi* por 2 h, y se realizó el ensayo de viabilidad para *T. cruzi*. Los Mφ MGL1$^{-/-}$ tuvieron mayor cantidad de parásitos viables internos (Fig. 10). Sin embargo, cuando los Mφ MGL1$^{-/-}$ son estimulados con IFN-γ estos son capaces de eliminar los parásitos internos no mostrando diferencias significativas con el grupo WT. Contrariamente, cuando los Mφ MGL1$^{-/-}$ fueron estimulados con LPS o con un doble estimulo de IFN-γ+LPS se observó una menor viabilidad de parásitos internalizados que los mostrados por los Mφ WT. Estos resultados muestran que la ausencia de MGL1 favorece la infección y proliferación intracelular de *T. cruzi*, lo cual sugiere que la ausencia de MGL1 podría repercutir en una deficiente activación. Así mismo, los Mφ MGL1$^{-/-}$ no eliminaron a los parásitos internos después de estímulos fuertes de activación como son la combinación del LPS+IFN-γ, el defecto para eliminar a los parásitos de *T. cruzi* parece ser independiente de las vías de activación de IFN-γ pero no de LPS.

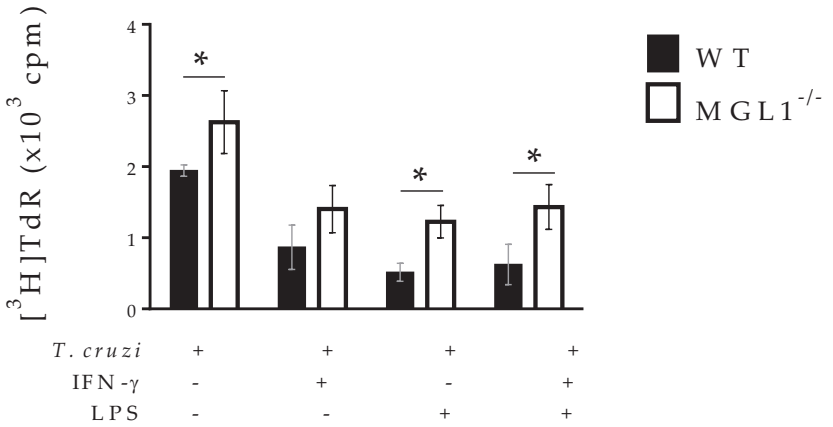


Figura 10. Viabilidad de parásitos provenientes de Mφ infectados *in vitro*. Mφ de ratones WT y MGL1$^{-/-}$ infectados *in vitro* con *T. cruzi,* proporción de 1:10 (Mφ/parásitos) y estimulados con IFN-γ (1μg/ml) y LPS+IFN-γ (LPS-0.2μg/ml; IFN-γ-1μg/ml), a las 24 hrs post-infección. A) Número de parásitos en sobrenadante. B) Proliferación de parásitos internalizados en Mφ WT y MGL1$^{-/-}$ infectados *T. cruzi,* y lisados para determinar los parásitos internalizados por el método de incorporación de (^{3}H)TdR. * $p<0.05$.

MGL1 en Mφ aumenta el Estallido el Oxidativo durante la infección *in vitro* con *T. cruzi*. Las especies reactivas de oxígeno (ROS) son producidas por procesos de oxidación dentro de los Mφ contra parásitos intracelulares, formando uno de los principales mecanismos antimicrobianos que participan en la defensa del huésped [73-75]. Nosotros quisimos saber si MGL1 favorece la producción de ROS en Mφ para la eliminación de *T. cruzi*. Se utilizo *C. albicans* como control ya que se ha reportado que induce la producción de ROS en Mφ [76]. Se realizó la prueba de NBT en Mφ infectados con *T. cruzi*. Se observó que los Mφ MGL1$^{-/-}$ presentaron un menor porcentaje Mφ positivos para NBT que los Mφ WT infectados con *T. cruzi* (40% y 79.7% respectivamente) (Fig. 11 a-c). Nuestros resultados muestran que MGL1 es importante en la producción de ROS, mostrando un nuevo receptor en la activación de Mφ.

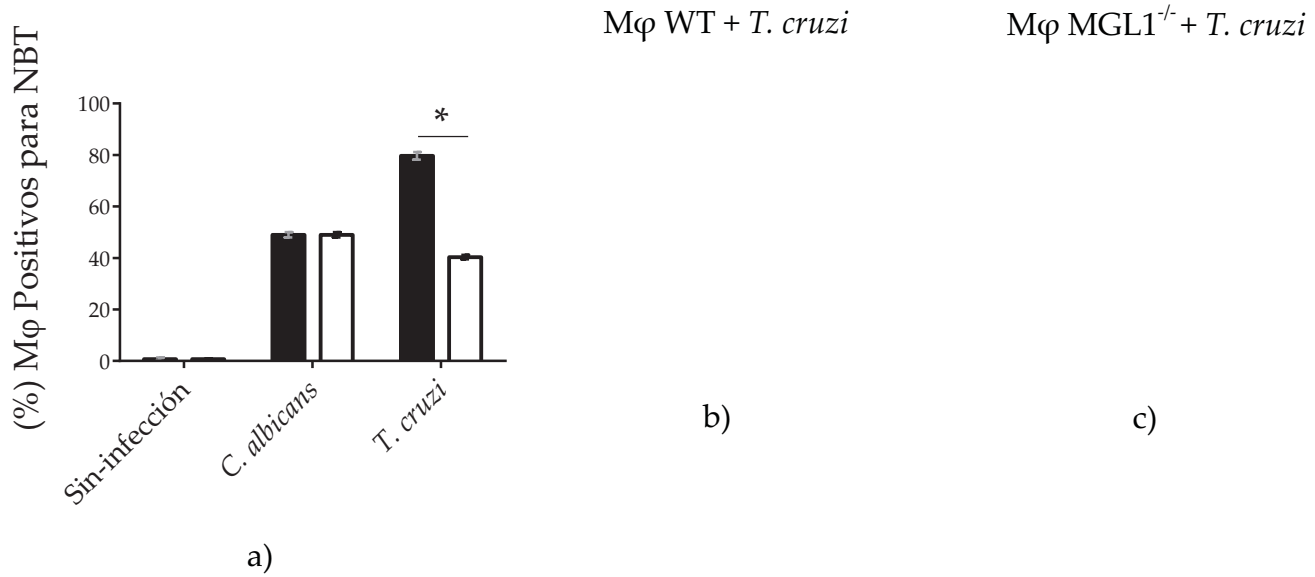


Figura 11. Producción Especies Reactivas de Oxigeno (ROS) por Mφ provenientes de ratones WT y MGL$^{-/-}$ infectados con *T. cruzi*. a) Mφ de ratones WT y MGL1$^{-/-}$ sin infección (Basal), infectados con *C. alibicans* (proporción 10:1), parásitos de *T. cruzi* (proporción 1:10) y estimulados Ag*Tc* (25μg) 40X. b) Mφ de ratones WT y MGL1$^{-/-}$ infectados con *T. cruzi* (proporción 1:10) 100X. *p<0.05 respecto al grupo Mφ WT.

MGL1 favorece la producción de óxido nítrico en Mφ infectados *in vitro*. Se ha reportado que el NO es una molécula importante en la eliminación de *T. cruzi* [77]. De los cultivos de Mφ infectados con *T. cruzi* (mencionado en la parte superior), se tomó el sobrenadante a las 24 h post-infección para cuantificar la producción de NO por la técnica de Greiss. Se observó diferencias en la producción de NO con un doble estimulo de IFN-γ+LPS siendo menor la producción en Mφ MGL1$^{-/-}$. Así mismo, se observa que cuando los Mφ MGL1$^{-/-}$ son infectados con *T. cruzi* tienen niveles significativamente menores de NO que los Mφ WT con los diferentes estímulos (Fig 12). Esta observación sugiere que el receptor de MGL1 participa en el reconocimiento de *T. cruzi* para la producción de NO. Nuestros resultados muestran que MGL1 actúa de manera sinérgica con la activación previa de IFN-γ (ya que se sabe que MGL1 reconoce a *T. cruzi*); de manera contraria no se observa este fenómeno con el receptor para LPS. En conclusión, la ausencia de MGL1 disminuye la producción de NO con la infección de *T. cruzi*.

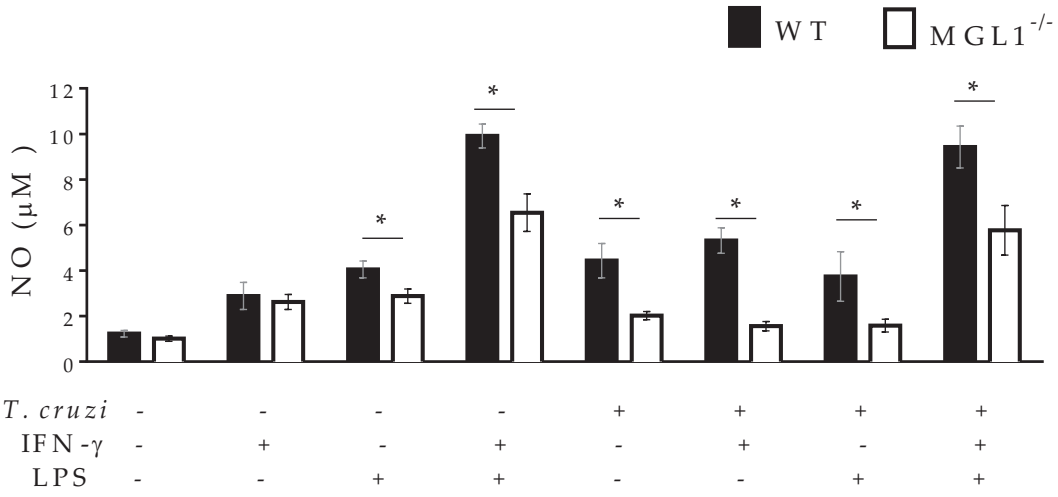


Figura 12. Concentración de oxido nítrico en el sobrenadante de Mφ de ratones WT y MGL1$^{-/-}$ infectados *in vitro* con *T. cruzi*. Mφ de ratones WT y MGL1$^{-/-}$ estimulados con IFN-γ (1µg/ml) y LPS+IFN-γ (LPS 100 ng/ml; IFN-γ-1µg/ml sin infección y con parásitos de *T. cruzi* (proporción 1:10). Gráfica de tres experimentos independientes, *p<0.05 respecto al grupo Mφ WT.

MGL1 es importante en la producción de IL-12, TNF-α e IL-10. La síntesis de las citocinas IL-12 y TNF-α por los Mφ es importante en control inicial de la infección por *T. cruzi* [78]. De manera contraria, la citocina anti-inflamatoria IL-10 es permisiva a la infección. Se cuantificó la concentración de IL-12, TNF-α e IL-10 en el sobrenadante de los cultivos de Mφ estimulados con IFN-γ LPS, IFN-γ+LPS, y con infección de *T. cruzi* por 24h. Los Mφ MGL1$^{-/-}$ estimulados con LPS tuvieron una disminución en la producción de IL-12 en comparación con Mφ WT. Mφ MGL1$^{-/-}$ estimulados con LPS y IFN-γ+LPS e infectados con *T. cruzi* tuvieron una menor producción de IL-12 y TNF-α en comparación con Mφ WT. Fue de llamar la atención que la producción de la citocina anti-inflamatoria IL-10 también mostro una disminución significativa en los Mφ MGL1$^{-/-}$ infectados con *T. cruzi* y infectados con *T. cruzi* estimulados IFN-γ y IFN-γ+LPS, comparados con los Mφ WT (Fig. 13). Estos resultados confirman una deficiente activación de los Mφ MGL1$^{-/-}$; y se sugiere que MGL1 está implicado en la expresión de los receptores de reconocimiento del LPS y/o en la activación de las vías de señalización para la síntesis de citocinas en proinflamatorias Mφ, como las MAPK cinasas.

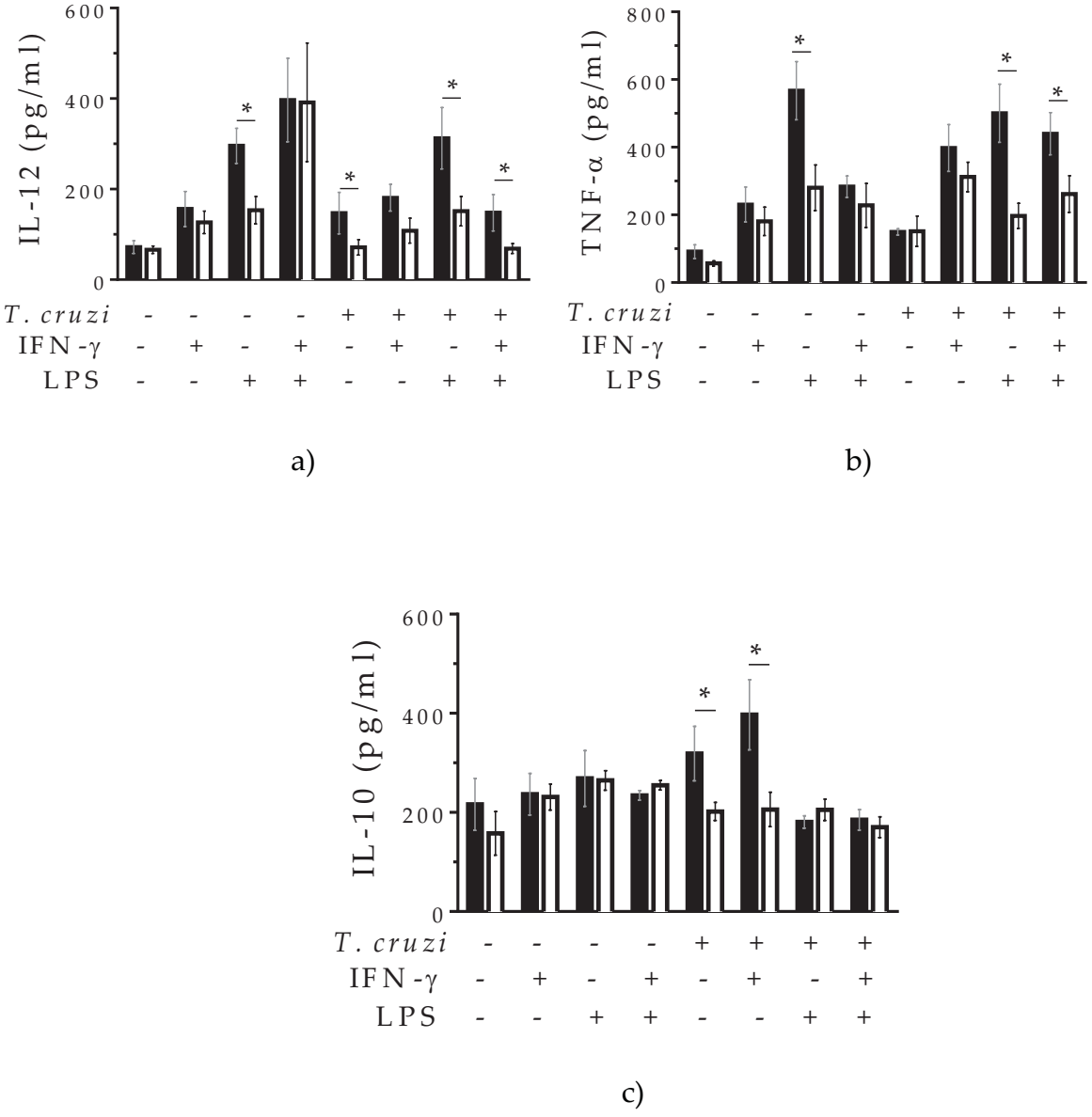


Figura 13. Concentración de las citocinas IL-12, IL-10 y TNF-α en el sobrenadante de Mφ ratones WT y MGL$^{-/-}$ infectados *in vitro* con *T. cruzi*. Mφ de ratones WT y MGL$^{-/-}$ infectados con *T. cruzi* (proporción 1:10). A) Concentración de IL-12. B) Concentración de TNF-α. C) Concentración de IL-10. Los gráficos son representativos de dos experimentos independientes, *p<0.05 respecto al grupo Mφ WT.

MGL1 favorece la expresión de TLR-2 y TLR-4 en Mφ la infección por *T. cruzi*. En Mφ los receptores TLR-2 y TLR-4 reconocen PAMPs en *T. cruzi* y este reconocimiento favorece activación de mecanismos trypanocidas [79, 80] [81]. Con el propósito de determinar si la ausencia de MGL1 afecta la expresión de TLR-2 y TLR-4; Mφ WT y MGL1$^{-/-}$ se estimularon con LPS, Ag*Tc* (Antígeno de *T. cruzi*) y *T. cruzi*. Los Mφ WT no presentaron diferencias significativas en el porcentaje de expresión de TLR-2 y TLR-4 sin estimulo en comparación con Mφ MGL1$^{-/-}$. Los Mφ WT tuvieron un mayor porcentaje de Mφ positivos para TLR-2 con los estímulos de LPS alrededor del 61.8%, mientras que los Mφ MGL1$^{-/-}$ mostraron un porcentaje de expresión de TLR-2 de 48.9% frente al estímulo de LPS. Pero no se observaron diferencias en la expresión de TLR-4 con el estímulo de LPS. Estudios realizados con células epiteliales gástricas y Mφ del epitelio gástrico, mostraron una que la activación de TLR-4 por *Helicobacter pylori* (el cual presenta en su membrana plasmática grandes cantidades de LPS, ligando natural del TLR4); induce la co-expresión de TLR-2 [82]. De igual manera, Mφ WT con el estímulo Ag*Tc* y con la infección de *T. cruzi* mostraron un mayor porcentaje de Mφ positivos para TLR2 y TLR-4, mostrando diferencias significativas con el grupo MGL1$^{-/-}$ (Fig. 14). Los resultados observados en nuestra investigación sugieren que la ausencia de MGL1 afecta la expresión de TLR-2 y TLR-4 frente a la infección con *T. cruzi*; esto se ve relacionado con una disminución en la producción de citocinas de tipo pro-inflamatorias como IL-12 y TNF-α, así como anti-inflamatorias (IL-10). Y probablemente esto impacte en la capacidad de los Mφ MGL1$^{-/-}$ como CPAs para activar de manera eficiente la respuesta celular T (tipo Th1) importante en el control de la infección por *T. cruzi*.

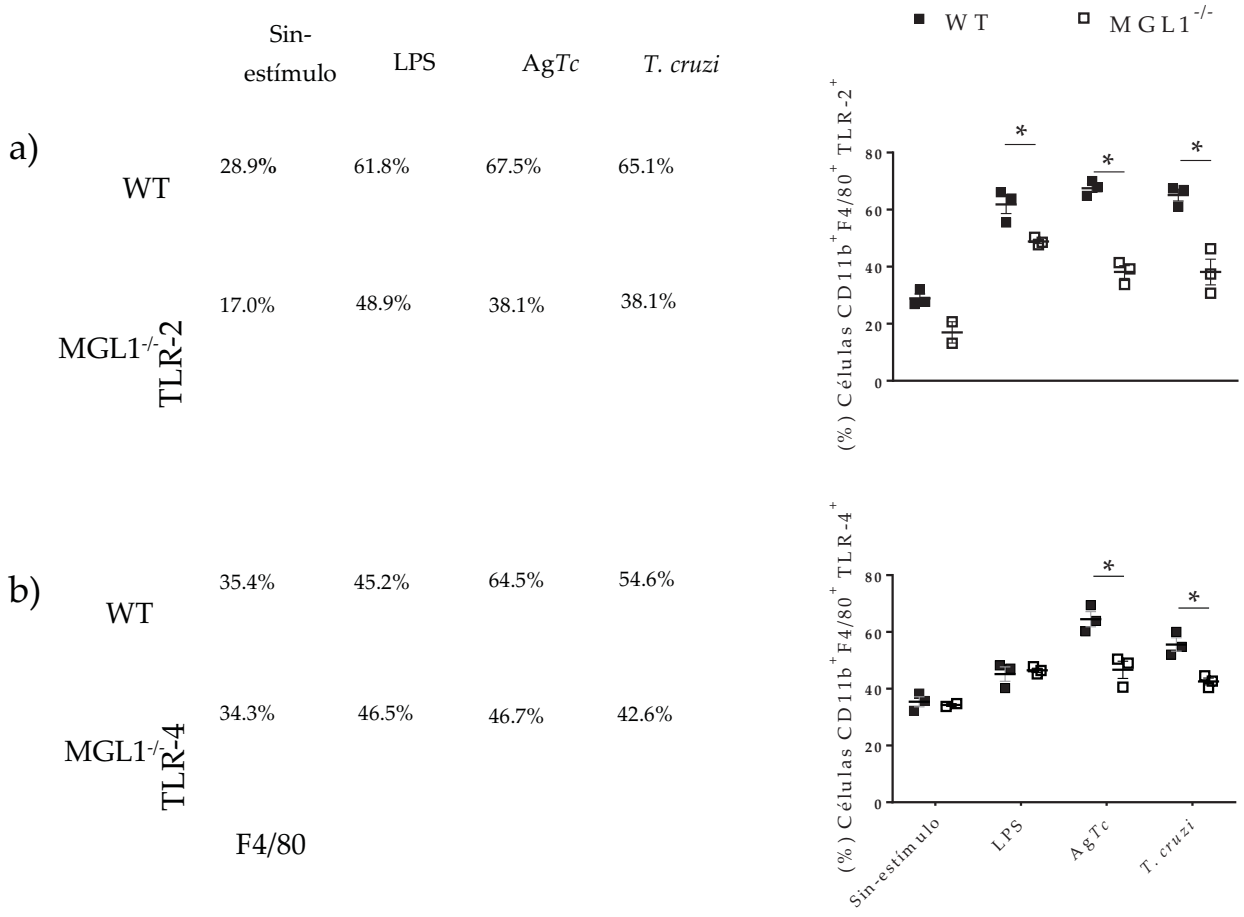


Figura 14. Expresión de TLR-2 y TLR-4 en Mφ MGL1$^{-/-}$ y WT estimulados con LPS, Ag*Tc* e infectados con *T. cruzi in vitro*. Mφ provenientes de ratones MGL1$^{-/-}$ y WT estimulados con LPS (100ng/ml) ó Ag*Tc* (25µg/ml) ó infección con *T. cruzi* (10 parásitos por 1 Mφ) por 24 h. (**a**) Dot plot de Mφ estimulados. (**b**) Grafica de barras del porcentaje de la expresión de TLR-2. Datos representativos de 2 experimentos. n=4. *p<0.05.

MGL1 participa en la expresión de MHC-II en la infección de *T. cruzi*. Diversos artículos han demostrado que los CLRs son receptores fagocíticos, además de participar en la presentación de antígenos a linfocitos (Lc-T) [47, 83, 84]; dentro de los receptores CLRs se encuentra el receptor para MGL [44]. Nosotros realizamos una activación de Mφ WT y MGL$^{-/-}$ *in vitro* con estímulos de LPS, Ag*Tc* y *T. cruzi* para observar la participación de MGL en la expresión de MHC-II y moléculas coestimuladoras. Se observaron diferencias significativas de la expresión MHC-II entre grupos con los estímulos con Ag*Tc* y *T. cruzi*, teniendo mayor expresión los Mφ WT, esto se relaciona con la deficiencia de los Mφ MGL$^{-/-}$ para la eliminación del parasito. Así mismo, se observaró una mayor expresión de CD40 en los Mφ WT con el estímulo de LPS y *T. cruzi*, esto podría deberse a que la expresión de esta molécula requiere que el parasito se encuentre vivo. Estos resultados muestran que MGL es importante en la expresión de MHC-II, y esta se ve implicada con la inducción de una respuesta inmune celular T, importante en contra la enfermedad de Chagas crónica.

		Sin-estímulo	LPS	Ag*Tc*	*T. cruzi*
WT	MHC-II	38.0%	36.5%	82.1%	64.0%
MGL1$^{-/-}$	MHC-II	48.9%	39.7%	59.6%	51.2%
WT	CD40	30.4%	70.4%	60.6%	67.4%
MGL1^{-}	CD40	33.2%	48.9%	51.9%	47.9%
WT	CD80	52.9%	63.3%	62.7%	61.9%
MGL1$^{-/-}$	CD80	48.3%	53.4%	62.8%	68.1%

F4/80

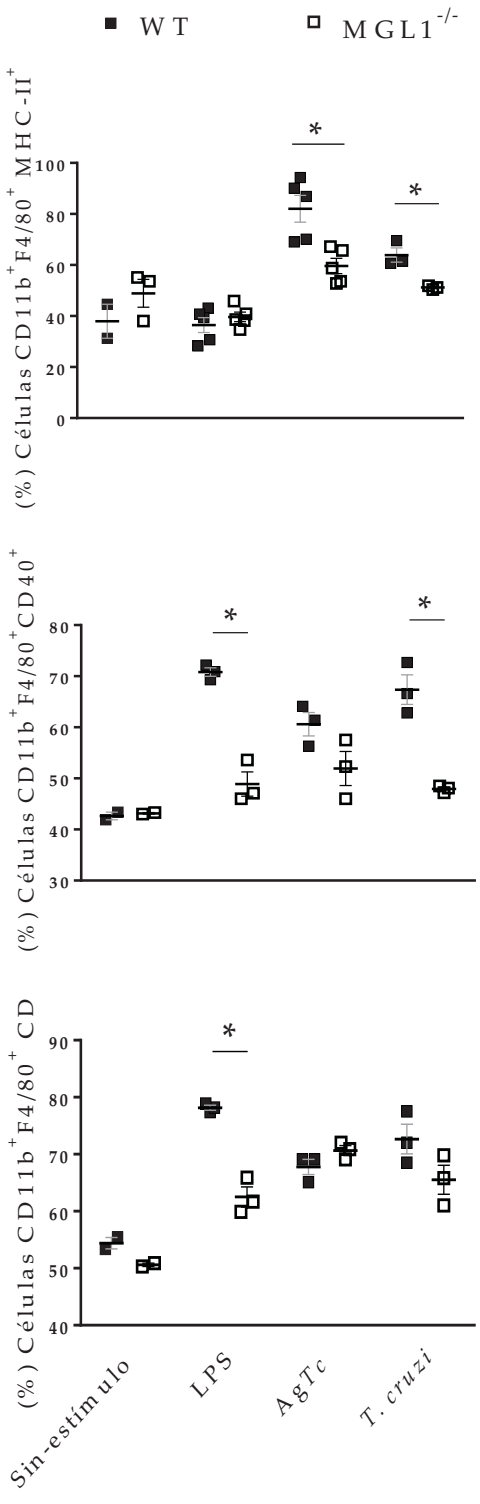


Figura 16. Expresión MHC-II y moléculas co-estimuladoras CD40 y CD80 de los Mφ estimulados con LPS, Ag*Tc* y *T. cruzi*. Mφ WT ó MGL1$^{-/-}$ Gráficos representativos de dos experimentos independientes. Mφ estimulados con Ag*Tc* (25μg/ml) ó LPS (100ng/ml) ó *T. cruzi* (1 Mφ/10 parásitos) y por 24 h.* $p < 0.05$.

Los co-cultivos Mφ MGL1$^{-/-}$ con linfocitos T de ratones WT tienen disminuida la producción de citocinas. Para determinar si MGL1 en Mφ participa en la activación y linfocitos T, se realizó un sistema de presentación de antígeno *in vitro* de Mφ WT y Mφ MGL1$^{-/-}$ con esplenocitos provenientes de una infección previa de *T. cruzi*. Se midió la concentración de TNF-α e IFN-γ en sobrenadante de ya que son estas importantes en la diferenciación de Linfocitos T hacia una respuesta inflamatoria-Th1, confiriendo protección en la infección de *T. cruzi.* TNF-α es conocido por sus propiedades de activación de Mφ citotóxicos productores de NO, de igual manera, IFN-γ como molécula derivada de linfocitos Th1 efectores y NKs, células involucradas en la destrucción del parásito [85]. Se midió la concentración en sobrenadante de IL-4 e IL-13 junto con la IL-10 ya que son citocinas de una respuesta anti-inflamatoria Th2, son antagonistas de la respuesta protectora Th1, tienen un papel crítico, limitando la respuesta inmune inflamatoria-Th1 hacia los patógenos para prevenir el daño sistémico del hospedero [86]. Se observó una producción significativamente disminuida de TNF-α en el co-cultivo de Mφ MGL1$^{-/-}$ estimulados con Ag*Tc* en comparación con los co-cultivos con Mφ WT estimulados con Ag*Tc*. La producción de citocinas derivadas de la activación de linfocitos mostro una menor concentración de IFN-γ, IL-4, IL-13 e IL-10 en los co-cultivos de Mφ MGL1$^{-/-}$ estimulados con Ag*Tc*. Estos resultados muestran una deficiente producción de las citocinas ambos perfiles, tanto del perfil inflamatorio como anti-inflamatorios en los co-cultivos de los Mφ MGL1$^{-/-}$ estimulados con Ag*Tc* con esplenocitos WT. Esto podrían deberse a una activación deficiente de los Mφ MGL1$^{-/-}$ favoreciendo una disminución de la activación de los linfocitos T. Un reporte refiere que ratones mMGL$^{-/-}$ son altamente susceptibles a la infección por el helminto *Taenia crassiceps*, asociada a una reducción significativa de los niveles de las citocinas pro-inflamatorias IL-12, IFN-γ y TNF-α, así como en la producción de NO [87]. En nuestro estudio la ausencia de MGL1 en los Mφ provoco una reducción de citocinas pro-inflamatorias y anti-inflamatorias significativa de los Linfocitos T, lo que concluye que MGL1 participar en la activación de los

Linfocitos T, esto se debe a que MGL1 favorece la expresión de MHC-II, así como, la activación de los Mφ por TLR-2 y TLR4, y la sucesiva producción de citocinas.

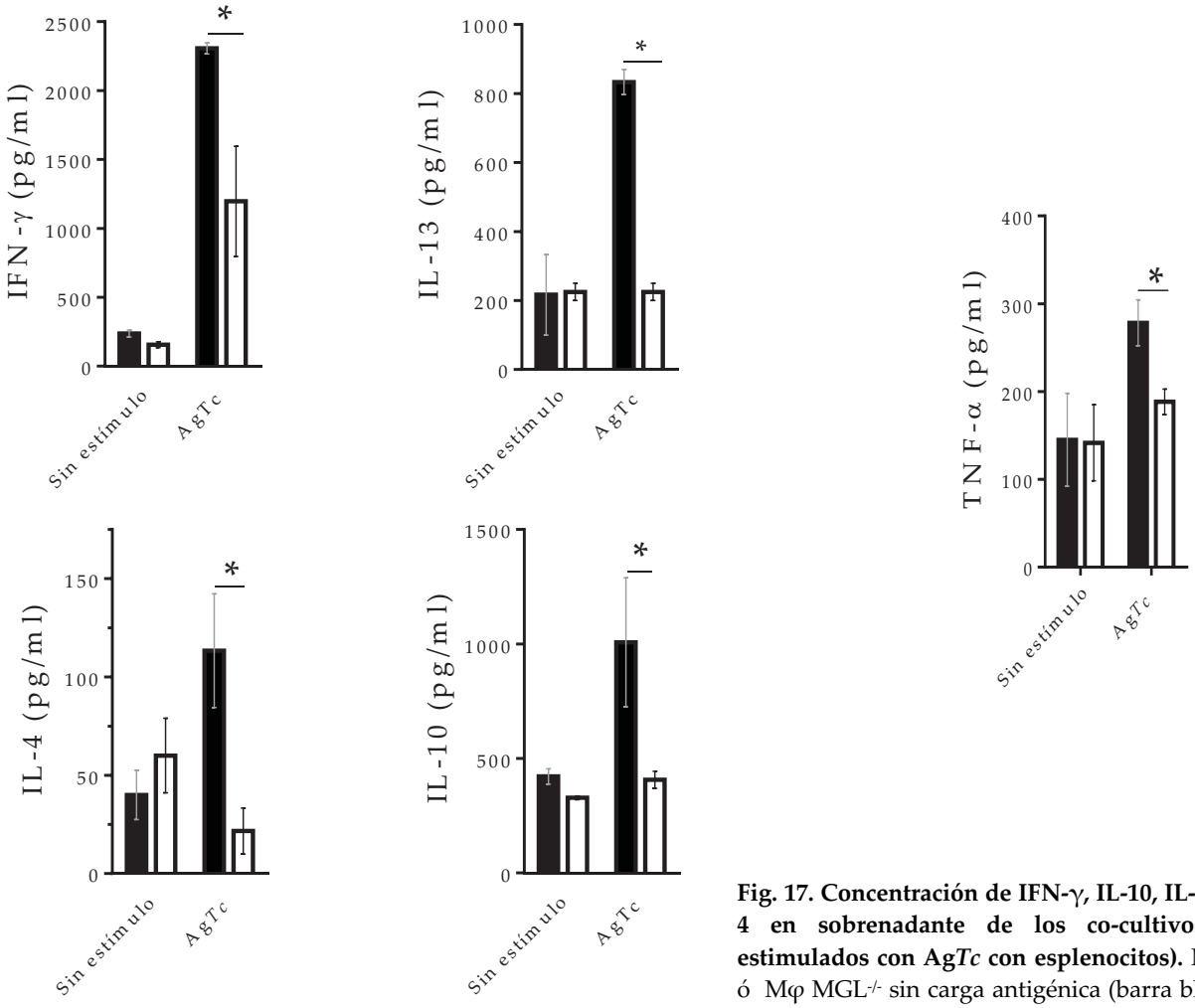


Fig. 17. Concentración de IFN-γ, IL-10, IL-13 e IL-4 en sobrenadante de los co-cultivos (Mφ estimulados con Ag*Tc* con esplenocitos). Mφ WT ó Mφ MGL$^{-/-}$ sin carga antigénica (barra blanca) ó estimulados con 2.5μg/ml de AgTc (barra negra) fueron co-cultivados con esplenocitos WT provenientes de una infección de *T. cruzi* de 21 días (relación 1Mφ:10 esplenocitos). A) ~~Producción~~ concentración de IFN-γ. B) Concentración de IL-10. C) concentración de IL-13. C) Concentración de IL-4. Gráficos representativos de dos experimentos independientes. *p<0.05 respecto a contraparte WT.

MGL1 en Mφ participa en la proliferación de linfocitos T de memoria. La expresión de la molécula del MHC-II se ve relacionada con una eficiencia de las CPAs en diversos procesos como: la previa lisis intracelular del parásito en los Mφ; la posterior carga de péptidos provenientes del parasito en MHC-II; así como, la expresión del MHC-II; en conjunto, con la expresión de moléculas co-estimuladoras favorece la activación de los linfocitos T. La proliferación de linfocitos T se midió por la técnica de incorporación de timidina tritiada. Se observó un incremento de en el valor de CPM del grupo de Mφ WT estimulados con Ag*Tc* y co-cultivados con linfocitos T (Mφ-AgTc-Lc) en comparación con lo Mφ MGL$^{-/-}$. El conjunto de estos resultados muestra que MGL1 participa en la fagocitosis, procesamiento y presentación de antígenos hacia linfocitos T, observándose una mayor producción de citocinas y una mayor proliferación de linfocitos T.

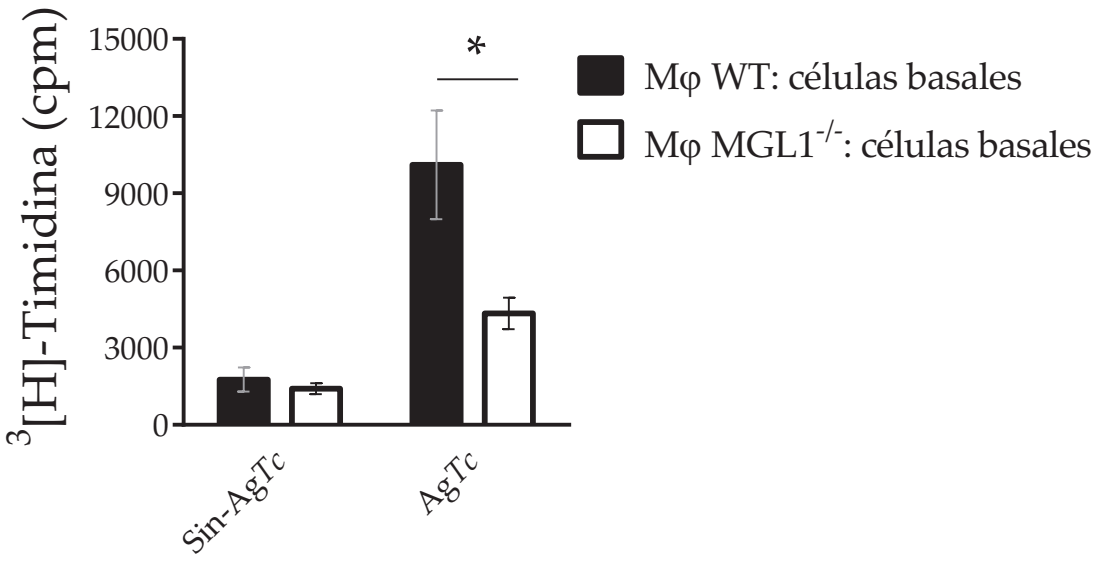


Fig. 18. MGL1 favorece una mayor proliferación de linfocitos T provenientes de una infección previa. Mφ WT ó Mφ MGL$^{-/-}$ 2.5µg/ml de AgTc, (Mφ-AgTc), Mφ co-cultivados con Linfocitos (Mφ-Lc y Mφ estimulados con AgTc y co-cultivados con Lc (Mφ-AgTc-Lc) . los linfocitos son provenientes de un ratón WT provenientes de una infección de *T. cruzi* de 21 días (relación 1Mφ: 10 esplenocitos). ** $p < 0.0028$.

Los Mφ diferenciados de Medula ósea de ratones MGL1$^{-/-}$ tiene una supresión del receptor MGL1. Ya que realizamos experimentos para determinar la participación de MGL1 en las vías señalización, necesitamos una mayor cantidad de Mφ, nosotros diferenciaremos Mφ a partir de medula ósea. Corroboraremos que los Mφ diferenciados de medula ósea tienen una supresión de MGL1. Reportes han mostrado que los Mφ diferenciados de medula ósea (BMMφ) expresan mayormente MGL1 que MGL2 [68]. Se midió por citometría de flujo la intensidad media de fluorescencia (IMF) de los anticuerpos específicos para MGL1 y MGL2 en Mφ (F4/80^{+}) estimulados *in vitro* por 24 ó 48 h con antígeno de *T. cruzi* (Ag*Tc*); los ratones WT mostraron una menor IMF MGL1 en niveles basales; cuando se estimulan con Ag*Tc* esta expresión aumenta, en Mφ diferenciados de medula ósea. Los Mφ MGL1$^{-/-}$ no se observa una expresión de MGL1 a nivel basal como con estimulo con estimulo de Ag*Tc* en ambos tipos de Mφ (tomando como referencia el isotipo). Además, se observó la presencia de MGL2 en ratones MGL$^{-/-}$ en estado basal en niveles iguales a los ratones WT (Fig 19. a y b).

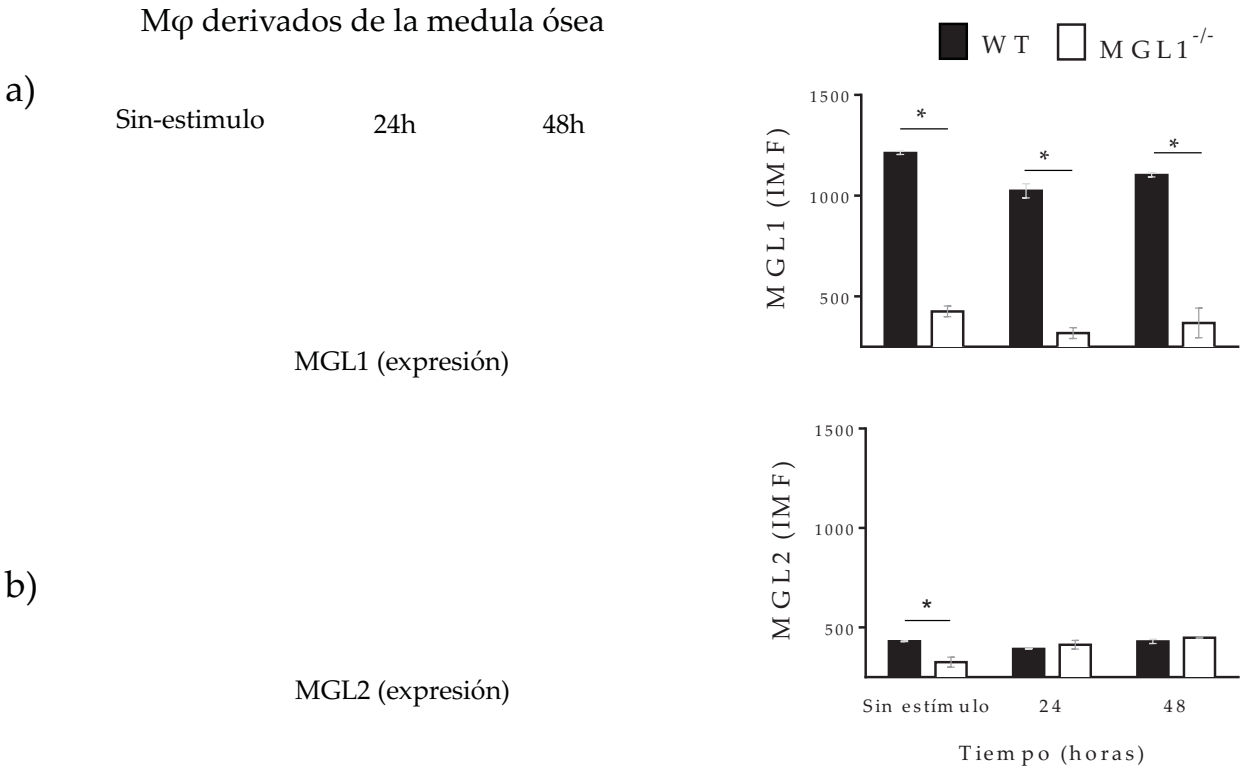


Figura 19. Expresión de MGL1 y MGL2 en Mφ diferenciados de medula ósea de ratones WT y MGL$^{-/-}$. (**a-b**) histograma de la expresión de MGL1 y MGL2 en Mφ infectados *in vitro* con *T. cruzi,* línea punteada isotipo; línea negra Mφ de ratones MGL1$^{-/-}$; área gris Mφ de ratones Wild-Type (WT). Gráfico de barras de la intensidad media de florecencia (IMF) Mφ que expresan MGL1 y MGL2. n=6 ratones por grupo. *=p<0.05.

MGL1 participa en la activación de NF-κB, c-jun y ERK1/2. Estudios recientes han demostrado que los CLRs tienen un papel importante en la activación celular. Los CLRs tiene motivos de señalización intracitoplasmática, que estos pueden inducir respuestas fagocítica, antimicrobianas así como la producción de citocinas que son protectoras o producen susceptibilidad contra microbios [88-90]. Nos preguntamos si MGL1 podría promover una vía de señalización que participara en la producción de citocinas y NO. Las vías de señalización más estudiadas para la producción de citocinas y NO son P38, ERK1/2, y los factores de transcripción AP-1 (c-jun) y NF-κB [89, 91, 92]. Se utilizaron Mφ diferenciados de médula ósea de ratones WT y MGL1$^{-/-}$, se estimularon con LPS y Ag*Tc* a 5 ", 15" y 30". No se observaron diferencias significativas en la activación de P38 entre grupos. De manera contraria; los Mφ MGL1$^{-/-}$ no mostraron activación en ERK1/2 después de la estimulación con Ag*Tc* o LPS en, mostrando de esta manera que MGL1 podría activar de manera directa a ERK1/2. De igual manera se observó una menor activación en c-jun y NF-κB en el estimulo a 5" y 30" y 5" y 15" (respectivamente) post-estimulación de Ag*Tc* en Mφ MGL1$^{-/-}$ (Fig. 20). Estos resultados muestran que MGL1 estimulado con Ag*Tc* juega un papel importante en la activación de ERK-1/2 además de una participación en la activación de c-jun y NF-κB.

a)

b)

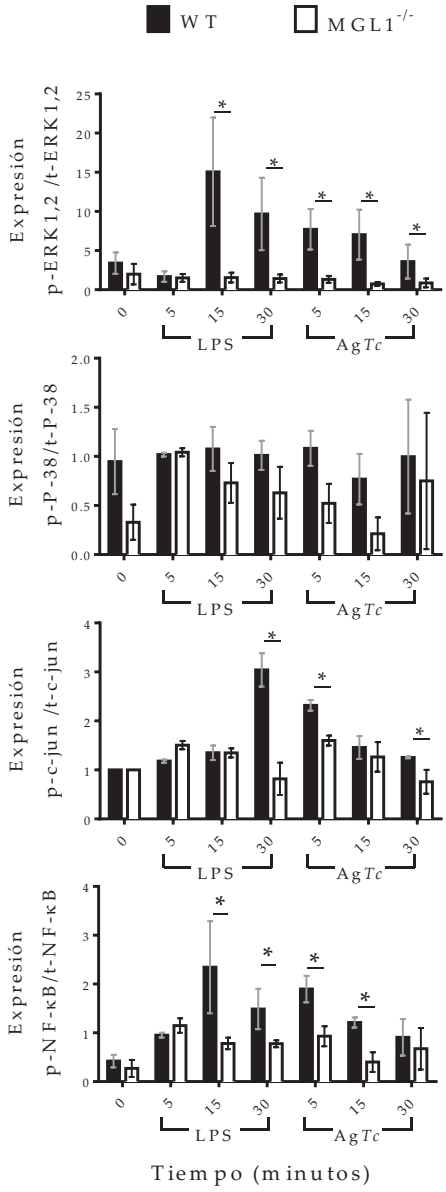


Figura 20. MGL1 ayuda en la activación de ERK1-2, c-jun y NF-κB. A) Western blot de P-38, NF-κB, c-jun y ERK-1/2 en BMMφ MGL$^{-/-}$ y WT estimulado con Ag*Tc* (25 μg/ml) o LPS (200 ng/ml) y estimulado por 5", 15", y 30"(p-, fosforilados; t-, totales). Se lisaron las células y se separaron 15 μg de extracto de proteína de célula completa mediante SDS-PAGE al 10% y se transfirieron usando los anticuerpos indicados. Se usó el anticuerpo GAPDH como control de carga. Los datos mostrados son representativos de dos experimentos independientes. Gráfico de barras del porcentaje de la expresión doble de P38, NF-κB y ERK1/2 BMMφ. Datos representativos de 2 experimentos n = 5 * P <0.05.

MGL1 juega un papel importante en la activación del inflamasoma. Uno de los principales factores que desencadenan la inflamación aguda o crónica es el inflamasoma [93]. NLRP3, es uno de los inflamasomas más importantes involucrados en el desarrollo del microentorno inflamatorio a través de la activación de caspasa1 produciendo la secreción de IL-1β, creando un microentorno inflamatorio [94]. Debido a que Mφ MGL1$^{-/-}$ expuestos a LPS o Ag*Tc* exhibieron una producción disminuida de citocinas pro-inflamatorias y esto se correlaciona con una deficiente activación de c-jun ERK1/2 y NF-κB, exploramos si Mφ MGL1$^{-/-}$ muestran deficiente activación del inflamasoma NLRP3. Se utilizaron Mφ WT y MGL$^{-/-}$ derivados de médula ósea, se estimularon con LPS, Ag*Tc* o LPS/Ag*Tc* durante 24 h y se realizó un análisis por Western blot la expresión de la proteína NLRP3. Como se muestra en la Figura 21a, b; Mφ WT estimulado con LPS, Ag*Tc* o LPS/Ag*Tc* aumentaron la producción de la proteína NLRP3 en comparación con los de las células no estimuladas. Es importante destacar que los Mφ MGL1$^{-/-}$ en comparación con Mφ WT no mostraron ningún aumento en los niveles de proteína NLRP3 tras la estimulación con LPS/Ag*Tc* o cualquiera de los estímulos solo. Con el fin de confirmar que la actividad del inflamasoma se altera en ausencia de MGL1, medimos la expresión de pro-caspasa1, caspasa1, por western blot; encontramos que tiene una existe una activación de caspasa1 en Mφ WT, y no se observa una expresión de caspasa1 cuando son doblemente estimulados (LPS/Ag*Tc*), y se observa una menor expresión de caspasa1 en Mφ MGL1$^{-/-}$ con los diferentes estímulos. De igual manera, la producción de IL-1β en los sobrenadantes de los mismos cultivos Mφ se observó niveles más altos en los Mφ WT estimulados con LPS, Ag*Tc* o LPS/Ag*Tc*, en contraste, Mφ MGL1$^{-/-}$ estimulado con cualquiera de los estímulos mostró niveles significativamente más bajos de producción de IL-1β que WT Mφ expuesto a LPS o Ag*Tc*. Estos resultados muestran que MGL1 es importante para la producción de NLPR3, pro-caspasa1 y IL-1β.

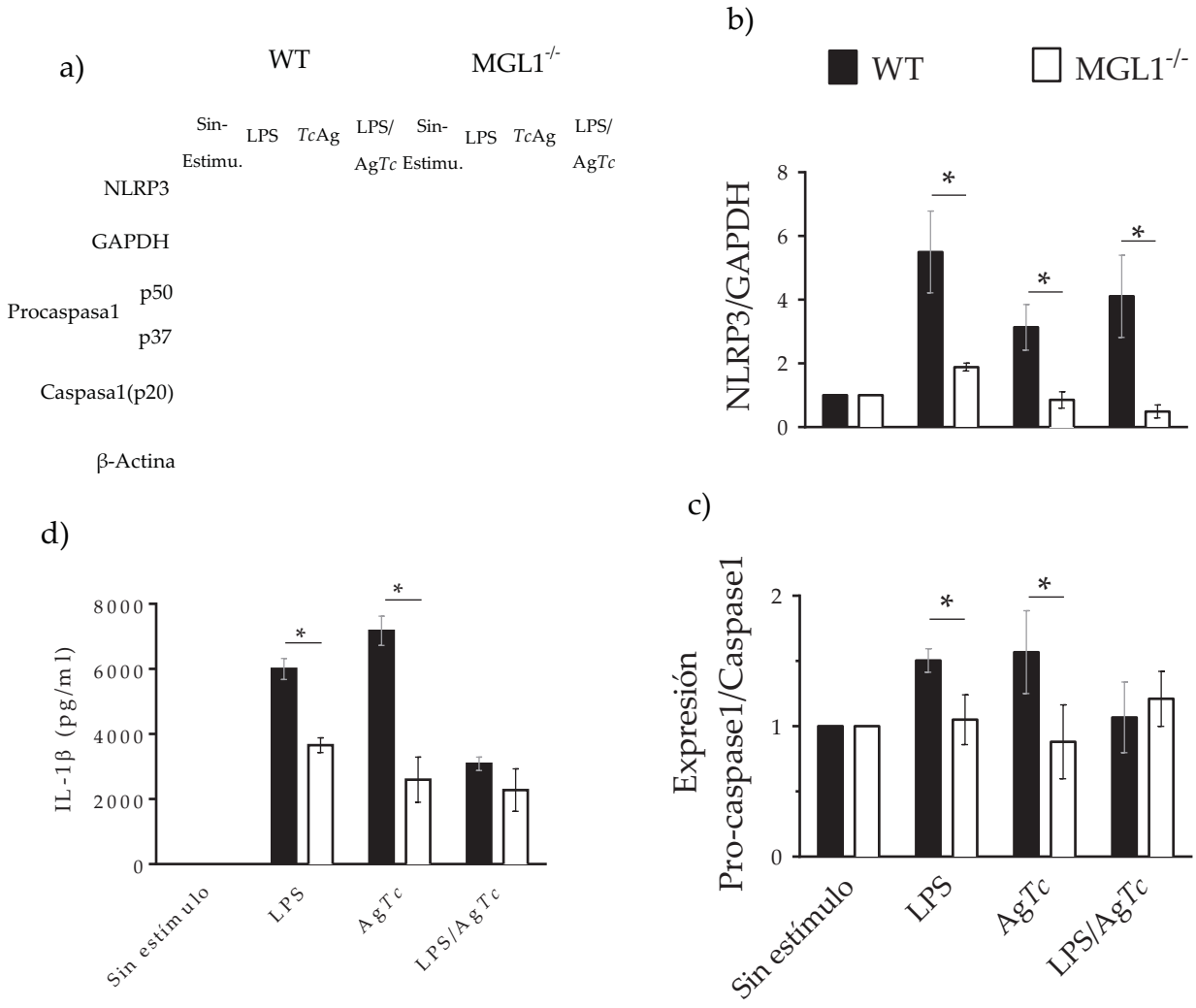


Figura 21. MGL1 regula a la baja la producción de NLRP3, Caspasa1 y IL-1β. (**a**) WT y MGL1$^{-/-}$ Mφ derivado de la médula ósea se estimularon durante 24 h con LPS (100 ng/ml) o Ag*Tc* (25 μg /ml) o LPS + Ag*Tc* (100 ng+25 μg/ml), en lisados de proteínas totales de Mφ se midieron la proteína NLRP3 por Western blotting. Transferencia de Western que muestra la regulación negativa de la expresión de la proteína NLRP3 en MGL1$^{-/-}$ Mφ. Y el análisis de densitometría para el Western blot NLRP3. (**b**) Cuantificación de IL-1β en el sobrenadante de cultivo celular por ELISA. Los datos de transferencia Western se normalizaron para el control de GAPDH y son representativos de dos experimentos separados. Los datos se representan como los medios (+ SEM), n = 5, * p <0.05.

Discusión

Los CLR tienen un papel importante en el reconocimiento de glicoconjugados parasitarios [54]. Estudios identifican a los CLR como una importante familia de PRRs que participan en la inducción de perfiles de expresión génica específicos contra patógenos, mediante la modulación de la señalización de TLR o induciendo directamente la expresión génica [89, 95, 96]. Nuestra investigación muestra la importancia de MGL1 para la activación celular y subsecuentemente la destrucción de *T. cruzi*. Resultados previos realizados en nuestro laboratorio mostraron la participación de MGL1 en la resistencia contra la infección intraperitoneal por *T. cruzi*, mostrando que los ratones MGL1$^{-/-}$ desarrollaron mayor parasitemia y mortalidad que los ratones WT [64]. En el presente trabajo mostramos resultados que describen la importancia de MGL1 en Mφ y su participación en la respuesta inmune innata contra *T. cruzi*.

Los Mφ tienen tres funciones principales; 1) fagocitosis, 2) inmunomodulación (a través de la oxidación intracelular y la producción de diversas citocinas) y 3) Presentación del antígeno [97]. Estudios han descrito a MGL1 como una molécula que participa en la internalización de antígeno [98]. Nuestros resultados muestran que los Mφ MGL1$^{-/-}$ tenien un mayor número de parásitos internos viables, pero la ausencia de este no limita la internalización de *T. cruzi*. En este trabajo no tenemos evidencias de que los Mφ fagocitaron a los epimastigotes, porque no centramos nuestro estudio en esta investigación. Sin embargo, creemos que los epimastigotes pueden invadir los macrófagos en cultivo por la vía de la fagocitosis clásica, mientras que los pocos tripomastigotes pueden invadir los macrófagos a través de un mecanismo activo, como se indica a continuación. El mecanismo clásico de infección celular de *T. cruzi* se refiere a que pocas horas después de que los tripomastigotes invaden las células, se transforman gradualmente en amastigotes (a través de una etapa intermedia replicativa tipo epimastigote).

Los amastigotes se dividen por fusión binaria y se diferencian nuevamente en tripomastigotes, que se liberan después de la lisis de la célula huésped. Además de este proceso clásico, la invasión activa de epimastigotes y amastigotes en las células también se ha informado [99, 100]. El mecanismo de invasión de las células fagocíticas y no profesionales por *T. cruzi* ha demostrado que la infección de la célula podría ser por: 1) fagocitosis o 2) por penetración activa del parásito [101]. La distribución de diferentes componentes de la célula huésped durante la invasión del parásito depende de las formas infecciosas y también de las células huésped, lo que ha sido demostrado por el reclutamiento de componentes de la matriz extracelular, receptores de integrina y elementos del citoesqueleto de las células HeLa y Vero [102] Barbosa y col. sugirieron que los parásitos pueden utilizar tanto la penetración activa como la fagocitosis típica para invadir los macrófagos y las células Vero, y que ambos procesos pueden ocurrir en la misma célula [103]. Se ha demostrado que los epimastigotes derivados de los cultivos de T. cruzi se fagocitan, mientras que las formas del torrente sanguíneo penetran activamente en los macrófagos [104]. Finalmente, es importante señalar que el vector insecto libera epimastigotes y tripomastigotes metacíclicos durante la defecación [105] lo que indica que ambas formas pueden ser infecciosas [100]. El mecanismo de invasión de *T. cruzi* en las células fagocíticas y no profesionales puede ser por: 1) fagocitosis o 2) por penetración activa del parásito[106]. La distribución de diferentes componentes de la célula huésped durante la invasión del parásito depende de las formas infecciosas y también de las células huésped, lo que ha sido demostrado por el reclutamiento de componentes de la matriz extracelular, receptores de integrina y elementos del citoesqueleto de las células HeLa y Vero [107]. De Souza y col. sugirió que los parásitos pueden utilizar tanto la penetración activa como la fagocitosis típica para invadir los macrófagos y las células Vero, y que ambos procesos pueden ocurrir en la misma célula [103]. Se ha demostrado que los epimastigotes derivados de los cultivos de *T. cruzi* se fagocitan, mientras que las formas del torrente sanguíneo penetran activamente en los macrófagos [104].

Finalmente, es importante señalar que el vector insecto libera epimastigotes y tripomastigotes metacíclicos durante la defecación [105], lo que indica que ambas formas pueden ser infecciosas.

Dirigimos nuestros esfuerzos para dilucidar que función tiene MGL1 en la producción de especies reactivas de oxígeno (ROS), óxido nítrico (NO), ya que uno de los principales mecanismos de eliminación de los parásitos internalizados en Mφ [98, 108]. Nuestros resultados demostraron que MGL1 promueve la oxidación intracelular en Mφ; esta mediante la producción de ROS, posiblemente es debido a que MGL1 la induce presión de TLR- 2 y TLR-4 durante la infección por *T. cruzi in vitro*. Se sabe que moléculas presentes en *T. cruzi* pueden activar a los Mφ, como el GIPL que es reconocido por el homodímero TLR-4, mientras que los GPI que contienen alquilacilglicerol es agonistas de TLR-2/6 [81, 109]. Estudios demostraron la participación de la señalización de TLR-4 en la producción de TNF-α y el óxido nítrico (NO), correlacionando el incremento de la expresión de TLR-4 con menores niveles de parasitemia de *T. cruzi* [110]. Así mismo, ratones C3H/He (TLR-4 no responsivos) presentaban una infección crónica de *T. cruzi* a pesar de la expresión aumentada de ARNm de IFN-γ [111]. De igual manera, Usando células TLR-4/MD-2 CHO (forma no funcional TLR-4) y estimuladas con GIPL de *T. cruzi* se demostró que estas no indujeron la activación del factor de transcripción NF-κB, causando una infección con cepa hipersusceptible de *T. cruzi* y una mortalidad más temprana que los ratones funcionales para TLR-4 (WT) [45]. Mostrando que el receptor TLR-4 tiene una función en el reconocimiento de *T. cruzi*, así como la activación de factores de transcripción y tanto la producción de citocinas, como la producción de NO son importantes para la resistencia contra *T. cruzi*.

De igual manera la expresión de TLR-2 es importante para la eliminación contra *T. cruzi*. Se ha demostrado que Mφ TLR-2$^{-/-}$ infectados por *T. cruzi in vitro* indujeron una menor producción de IL-12p40 y TNF-α que Mφ WT [45]. Nuestros estudios mostraron que MGL1 en Mφ es

importante en la expresión de TLR-2 y TLR-4. Estos estudios se ven sustentados con los realizados con células epiteliales gástricas en donde revelan que la señalización de TLR-4 iniciada por LPS de *Helicobacter pylori* induce la expresión de TLR-2, y ambos TLRs trabajan sinérgicamente para la inducción de iNOS [112]. Así mismo, se sabe que MyD88$^{-/-}$ es una molécula acopladora rio abajo de TLR-2 y TLR-4 que participa en la eliminación de *T. cruzi*. Mφ de ratones MyD88$^{-/-}$ mostraron una reducción en la fosforilación de ERK-1/2 y p38 siendo altamente susceptibles a la infección con *T. cruzi* en comparación con TLR-2$^{-/-}$ [113]. Todas estas investigaciones muestran que los TLRs tienen un papel importante en la síntesis de citocinas proinflamatorias, así como funciones efectoras durante la infección por *T. cruzi in vivo*. En adición con estos resultados, nuestros estudios concluyen que la activación sinérgica de MGL-TLR-4 induceda por LPS puede ayudar a la expresión cooperativa de TLR-2. Sin embargo, se desconoce si el aumento de ROS en WT Mφ es por la expresión de MGL o representa la expresión conjunta de MGL, TLR-2 y TLR-4.

Nuestra investigación destaca por los avances en la comprensión de los CLRs; en particular MGL1 y la cooperación con TLRs. Este estudio muestra a MGL1 como un regulador para la expresión positiva de los receptores TLR-2 y TLR-4, así como la secreción de IL-10 y TNF-α. Una activación combinada de varios receptores puede dar como resultado efectos sinérgicos o antagonistas que modulan la inmunidad innata y adaptativa [114]. Para dilucidar las vías de señalización que pueden estar involucradas nosotros utilizamos un doble estimulo de IFN-γ y LPS así como antígeno total de *T. cruzi* (Ag*Tc*), ya que se sabe que el IFN-γ y LPS activan de manera sinérgica por dos vías diferentes para la producción de iNOS [115]. Estudios muestran que NO se produce en Mφ de manera basal, y después de los estímulos con IFN-y+LPS estos niveles incrementa 20 veces más [116]. Nosotros mostramos que MGL ayuda potencializar la respuesta de LPS por la expresión de su agonista, TLR-4. Una comprensión completa del papel

de los TLRs en la resistencia del huésped contra la infección requiere la "descodificación" de estas múltiples interacciones de diversos receptores [117].

Nosotros proponemos que los próximos estudios a realizar será importante comprender las vías cooperativas entre los receptores TLR-CLR y el papel que ambos tiene en la formación de la inmunidad antimicrobiana, esto ofrecerá un gran potencial para el desarrollo futuro de terapias para la intervención de la enfermedad. Son pocos los estudios que ha demostrado tal cooperación, uno de estos trabajos, muestra la señalización a través de TLR2 y CLR (DC-SIGN) la cual puede mejorar y mantener la activación de ERK1-2 para la producción de citocinas [90].

Si bien reconocemos que la interacción temprana de los tripomastigotes sanguíneos con los macrófagos dicta en gran medida el resultado de la enfermedad y, por lo tanto, sería más informativo estimular los macrófagos con el antígeno derivado del tripomastigote, sin embargo, esta etapa proviene de la sangre de un ratón infectado, que creemos que es una desventaja porque los parásitos ya podrían ser opsonizados por anticuerpos o por opsoninas complementarias. Además, es bien sabido que los tripomastigotes expresan enzimas trans-sialidasa para enmascarar sus antígenos con ácido siálico derivado del huésped ([104]), esto último puede causar que los antígenos de glucano no estén adecuadamente expuestos en la superficie de este parásito. Por esta razón, decidimos exponer las BMM a epimastigotes derivados de cultivos que, previamente, hemos demostrado que expresan ligandos MGL-1 (galactosa y N-acetilgalactosamina) como se revela al teñir con la lectina jacalina [64]). Por lo tanto, la estimulación de los macrófagos BM con epimastigotes derivados de cultivos es un sistema confiable para estudiar los ligandos de carbohidratos derivados de tripanosoma para MGL1. Hemos agregado esta información en la sección de discusión.

En la actualidad los trabajos que describen de la importancia de los CLRs en las vías de señalización intracelular de activación o inhibición van en ascenso. Los CLRs mejor estudiados

son aquellos con motivos de activación basados en tirosina de inmunoreceptor (ITAM). Estos ITAM pueden ser un componente integral de la cola citoplásmica CLR (por ejemplo, dectin-1 y DNGR1) [118]. Se ha reportado que el receptor MGL1 presenta dominios intracelulares de tirosina, mostrándose una nula fosforilación del dominio de tirosina en Mφ MGL1$^{-/-}$ frente a la exposición de *T. crassiceps* en comparación con Mφ WT, asociándose la expresión de MGL1 con una mayor producción de TNF-α e IFN-γ en células de bazo provenientes de una infección de 4-8 semanas por *T. crassiceps* y antígeno soluble de *T. crassiceps* (TcSol) *in vivo* [87]. Así mismo, hMGL en DC inmaduras de donantes sanos humanos estimulados por el glucopéptido MUC19Tn (un glucopéptido con GalNAc) desencadenaron una la fosforilación de ERK1/2 y NF-κB, disminuyendo la fagocitosis [119]. Para dilucidar el papel de MGL murino en las vías de señalización, nosotros activamos Mφ WT y Mφ MGL$^{-/-}$ en tiempos tempranos (minutos) ya que se ha reportado que las diferencias significativas en la expresión de los TLR se dan después de 6 horas pos-estimulo [120]. Nuestros estudios muestran que MGL1 activa vías de señalización intracelulares en particular ERK1/2, c-jun y NFκB en minutos tras el estímulo con Ag*Tc*. Después de 24 h la expresión de TLR-2 y TLR-4 incrementa, funcionando de manera colaborativa con la expresión de MGL1 produciendo de NO, ROS y citocinas.

Otra vía de señalización que se ha descrito es la del CLR-Receptor de Manosa (MR), el cual se expresa en Mφ, este tiene un papel anti-infamatorio. Los ligando de MR son manosa, fucosa y N-acetilglucosamina presentes en *T. cruzi*, esta señalización regula negativamente el JNK, p44/p42 (ERK-1/2) y p38, aumentando la infección por *T. cruzi in vivo* [121]. Así mismo, Ropert y colaboradores evaluaron la capacidad de las glicoproteínas de trypomastigotes de *T. cruzi* (tGPI-mucina) en Mφ inflamatorios, mostrando una activación de ERK-1/2, proteína quinasa activada por estrés (SAPK), quinasa-1/proteína cinasa activada por mitógeno (MAPK) quinasa-4 y p38/SAPK -2, (entre 15 y 30 min); mostrando que estas moléculas tiene una posible participación en la activación del TNF-α y la síntesis de IL-12 [122]. Nuestros resultados abren

la posibilidad que la activación de ERK-1/2 mostrada por Ropert de Mφ con el estímulo de AgTc (tGPI-mucina) se deban a un previo estimulo de MGL.

Nosotros pensábamos que la posible vía que activación de MGL1 rio abajo es Raf /MEK /ERK-1/2. Estas hipótesis es sustentada por el estudio realizado por Terrazas y colaboradores, el cual reporto que los receptores MGL, MR y TLR-2 tienen un papel en el reconocimiento de antígenos excretados/secretados por *T. crassiceps* (TcES) y estos activan la fosforilación de cRAF[123]. De igual manera, se sabe que la cascada de ERK-1/2 generalmente se inicia mediante la activación de proteínas G (como Ras), que reclutan las quinasas Raf de la capa a la membrana plasmática[124]. Así mismo, estudios reportan que la rio arriba de ERK-1/2 se encuentra Raf, además la vía Raf-ERK, estos puede activar factores de transcripción rio abajo como AP-1 (c-jun), c-Myc y Ets-1 para la producción de citocinas [125]. Nuestros datos concluyen que MGL1 tiene un papel directo en la activación de ERK1/2 ya que observamos un abatimiento de esta molécula en Mφ MGL1$^{-/-}$, sugiriendo indirectamente que MGL1 tiene una respuesta antagónica a MR frente a *T. cruzi*.

Así mismo, nosotros mostramos que MGL1 juega un papel en potencializar la activación de NFκB para la producción de citocinas, así como NO. Estudios han mostrado que el bloqueo de NFκB, impidiendo la translocación nuclear de p65 y la actividad de unión de NFκB p65/ADN mostraron una significativamente inhibición de NO, iNOS, así como ROS; además de disminuir la producción de proinflamatorias como IL-6, IL-1β y TNF-α, después del estímulo con LPS [126].De igual manera ERK1/2 se ve implicada en la producción de TNF-α; estudios han revelado que en células RAW264.7 al ser estimuladas con sauchinona (una molécula que aislada de *Saururus chinensis)* con un previo estimulo de LPS inhibía la fosforilación de c-Raf- MEK1/2 - ERK1/2, además de la activación de NFκB lo cual provoca una inhibición de la expresión de TNF-α inducida por LPS [127]. Estos estudios nos muestran de una manera clara que la

supresión de MGL1 en Mφ inhibe la activación de ERK-1/2 y disminuyen la activación de NFκB; y estas dos moléculas actúan en la síntesis de citocinas.

De igual manera la síntesis de iNOS se ve regulada por las vías MAPKs, así como factores de transcripción; dentro de estas vías de encuentra ERK-1/2 y NFκB. Estudios usando células RAW 264.7γNO(-) encontraron que la inhibición de la ruta de ERK-1/2 reduce la expresión de TNF-α y iNOS [115]. De igual manera otros estudios mostraron que el promotor de iNOS contiene dos elementos para NF-κB que cuando son inhibidos evitan la expresión de iNOS y la subsecuente producción de NO [128]. Nuestros resultados mostraron que MGL1 fosforila ERK1/2, c-jun y NF-κB esto provoca un aumento de NO. iNOS se expresó en Mφ por la exposición previa a LPS y citocinas proinflamatorias, como TNF-α e IFN-γ. La importancia de una activación temprana de los Mφ puede dirigir al éxito o al fracaso contra una enfermedad parasitaria. La activación de hMGL (MGL de humano) en monocitos mediante una unión de su ligando promueve un aumento de la producción de IL-10 y TNF-α para niveles elevados de ARNm de IL-10 y una mayor estabilidad del ARNm de TNF-α [63]. Esta activación temprana de Mφ conduce a la producción de citocinas proinflamatorias que son importantes para el control inicial de la infección contra *T. cruzi* y que mantienen la respuesta inmune adaptativa posterior [129].

Como lo mencionamos previamente, una de las principales funciones de las CPAs es la presentación del antígeno [130]. Los Mφ dirigen la respuesta inmune celular T por la selección clonal de linfocitos T específicos al MHC-II, y con esto dirigen la inmunidad adaptativa. Las células T tiene un papel importante en evitar el daño fisiopatológico de la enfermedad de Chagas. Se ha demostrado que el antígeno TcMuc presente en *T. cruzi* promueve la falta de respuesta de las células T $CD4^+$ en ratones, ocasionando la disminuyendo la producción de IFN-γ, IL-2, IL-4 e IL-10. Además, los ratones estimulados con TcMuc y posteriormente infectados con *T. cruzi* desarrollaron una parasitemia sanguínea creciente en comparación que el grupo sin el previo estimulo [131]. Sin embargo, cuando los ratones son tratados con el antígeno de *T.*

cruzi-GIPL, se observó un mayor porcentaje de linfocitos T $CD4^+$, $CD8^+$ y linfocitos B, que expresan el marcador de activación CD69 en comparación con los ratones C57BL/0ScCr $TLR4^{-/-}$ [132]. Mostrando así que el ligando TLR-4-GIPL es importante para la proliferación de linfocitos $CD4^+$, $CD8^+$ y B. De manera muy interesante, nuestros resultados muestran por primera vez el potencial que tiene MGL1 en la activación de linfocitos T específicos contra *T. cruzi*.

Investigaciones han demostrado la importancia de CD40-CD40L; en donde a epimastigotes (cepa Y) se les transfecto con el gen CD40L murino (pTEX-CD40L), cuando estos parásitos se inocularon en ratones se observó un nivel muy bajo de parasitemia y nula mortalidad (con la cepa YpTEX-CD40L). Además, la capacidad proliferativa y la secreción de IFN-α se preservaron en células de bazo (SC) de ratones infectados con YpTEX-CD40L pero no en el control (YpTEX). Estos resultados sugieren que el CD40L en la infección con *T. cruzi* transfectado está involucrado en la modulación de una respuesta inmune antiparasitaria[133]. Relacionando estos resultados con los nuestros, nosotros observamos que Mφ $MGL1^{-/-}$ infectados con *T. cruzi* expresaron una disminución en la expresión de CD40 y esto se relaciona con una mayor cantidad de parásitos internos en comparación de los Mφ WT.

Una de las proteínas intracelulares que se ha demostrado tener un efecto contra la infección de *T. cruzi* es el inflamasoma. Gonçalves y colaboradores demostraron que la infección por *T. cruzi* induce la producción de IL-1β de forma dependiente de NLRP3 y caspasa-1. Mostrando que los ratones $NLRP3^{-/-}$ y $caspasa1^{-/-}$ exhibieron un alto número de parásitos *T. cruzi* en sangre, con una magnitud de parasitemia pico comparable a los ratones MyD88-/- e $iNOS^{-/-}$; indicando que la participación del inflamasoma NLRP3 es importante en el control de la fase aguda de la infección por *T. cruzi*. Tomados en conjunto, nuestros resultados demuestran un papel para el inflamasoma NLRP3 en el control de la infección por *T. cruzi* e identificamos la producción de

NO mediada por NLRP3, dependiente de caspasa-1 e independiente de IL-1R como un nuevo mecanismo efector para estos receptores innatos [134]. la maquinaria general del inflamasoma es deficiente en los Mφ deficientes en MGL1. la expresión de Pro-caspase1/Caspase1, se reduce en las células MGL1$^{-/-}$, lo que significa que hay una mayor proporción de caspasa activa.

A la luz de los resultados proporcionados, esta sería la fuente más probable para la expresión reducida de IL-1ß madura. Hablar sobre la activación de la procaspasa 1 no es realmente precisa. Finalmente, creo que los resultados son indicativos de una expresión reducida de todos esos componentes.

El conjunto de nuestros resultados muestra que MGL1 tiene una función crítica contra *T. cruzi* favorece la expresión de TLR2 y TLR4 tiene un papel en la producción de ROS, NO y citocinas, desencadenando no solo la secreción mejorada de IL-10, sino también aumentó la producción de TNF-α después del reconocimiento concomitante de TLR y la unión de MGL-GalNAc. Así mismo mMGL mostró la capacidad de transportar estos antígenos a los y cargarlos a moléculas de MHC-II para luego ser presentados al TCR de los linfocitos T específicos y así poder activar los linfocitos T CD4^{+}.

Estamos evaluando la absorción de ^{3}H-timidina en epimastigotes que provienen de los amastigotes sobrevivientes después de la estimulación de macrófagos.

Parece contradictorio que en ausencia de MGL1 en macrófagos presenten más parásitos, si MGL era la forma en que los parásitos solían ser internalizados. En este trabajo no respondemos si MGL1 es la forma de internalización, encontramos que en ausencia de macrófagos MGL tienen una activación deficiente de algunos de los mecanismos tripanocíticos. Esta activación deficiente depende de la interacción de los receptores MGL1 con los antígenos glucosilados de este parásito.

La internalización de T. cruzi a través de MGL es un tema a resolver en el futuro. Por lo tanto, hemos modificado un poco nuestro modelo hipotético mediante la introducción de un signo interrogativo sobre el papel de MGL1 asociado con la fagocitosis de este parásito.

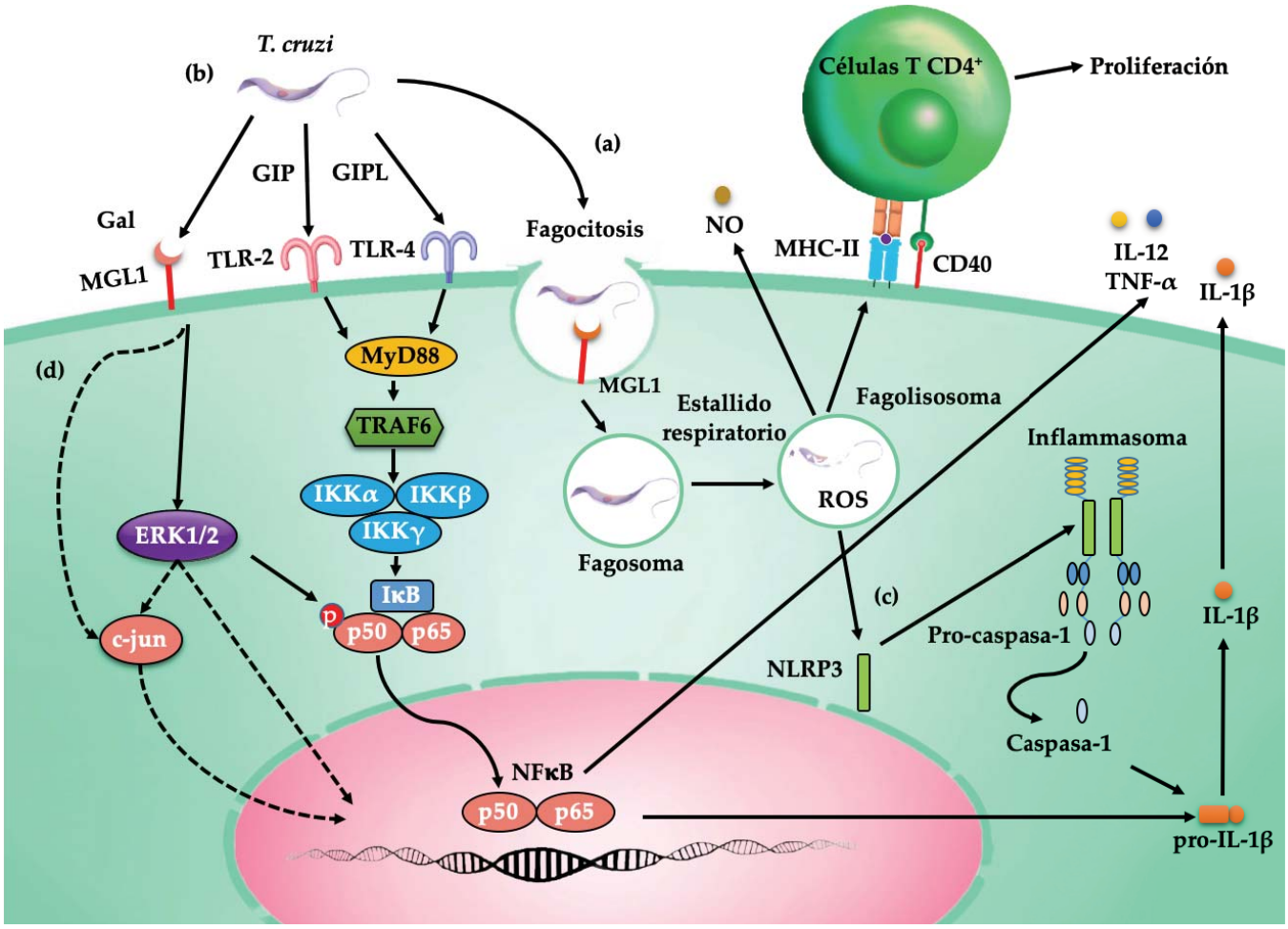


Figura 19. Papel de en MGL1 en Mφ contra *T. cruzi*. a) La unión de MGL1-*T. cruzi* produce la fagocitosis del parásito llevado al compartimiento fagocítico para su degradación en el fagolisosoma. Se genera la degradación del parásito para en la proteólisis intravacuolar ayudada por la previa generación de choque respiratorio así como ROS, generando péptidos que se unen a moléculas principales de MHC-II, que luego se desplazan a la superficie de la célula para mediar en la presentación de péptidos de *T. cruzi* a células T CD4 +. Las células T efectoras y de memoria proliferan después del reconocimiento de antígenos por el MHCII-TCR, presentes desarrollen la secreción de citocinas efectoras. b) Los ligados de *T. cruzi*, que incluyen glicolípidos, glicolpéptidos y proteínas, son reconocidos por los receptores MGL1 y TLR-2 y 4 para la generación de la activación que promueve la fosforilación a NF-κB, y dirige a ERK1-2 promoviendo las citocinas. Al mismo tiempo, MGL1 promueve la expresión de TLR-2 y 4, MHC-II y CD40. El receptor de lectina de tipo C MGL1 pueden desencadenar vías de señalización que resultan en la activación de las funciones celulares. Esta vía comienza cuando MGL1 reconoce a su ligando, y esto inducen vías de activación a través de motivos de activación basados en tirosina de inmunoreceptor (ITAM). La subsecuente señalización de ERK1/2. Así mismo, ERK-1/2 puede activar a los factores de transcripción c-jun y NF-κB, y de manera cooperativa las vías de MGL1-TLRs transcriben genes proinflamtorios. Se sabe los factores de transcripción como AP-1 (c-jun) y NF-κB aumentan la expresión de TLR-4 [135]. El promotor de TLR4 murino tiene sitios de unión de los diferentes factores de transcripción potencialmente AP-1, NFκB, STAT1 y GR, para la expresión de TLR4 inducida por TNFα[120]. La fosforilación y la acetilación de la subunidad p65 de NF-κB, aumentando de ese modo las respuestas proinflamatorias.

Conclusiones

- **La presencia de MGL1 reduce la cantidad de parásitos internos.**
- **La presencia de MGL1 y el estímulo de IFN-γ+LPS en la infección ayuda a una mayor producción de NO y ROS.**
- **MGL1 promueve la producción de citocinas.**
- **MGL1 frente a la infección de *T. cruzi* ayuda a la expresión de TLR-2 y TLR-4.**
- **MGL1 aumenta la expresión de MHC-II después del contacto con *T. cruzi*.**
- **MGL1 promueve la proliferación y producción de citocinas le linfocitos T de memoria.**

APENDICE I: ARTÍCULOS RELACIONADOS CON MGL Y LA INFECCIÓN POR *TRYPANOSOMA CRUZI.*

Articulo 1: Alicia Vázquez, Juan de Dios Ruiz-Rosado, Luis I. Terrazas, Imelda Juárez, Lorena Gomez-Garcia, Elsa Calleja1, Griselda Camacho, Ana Cháve1, Miriam Romero, Tonathiu Rodriguez1, Bertha Espinoza3 and Miriam Rodriguez-Sosa1. 2014. Mouse Macrophage Galactose-type Lectin (mMGL) is Critical for Host Resistance against *Trypanosoma cruz*i Infection. International Journal of Biological Sciences. IF 4.067

Int. J. Biol. Sci. 2014, Vol. 10 909

IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Biological Sciences

2014; 10(8): 909-920. doi: 10.7150/ijbs.9214

Research Paper

Mouse Macrophage Galactose-type Lectin (mMGL) is Critical for Host Resistance against *Trypanosoma cruzi* Infection

Alicia Vázquez[1], Juan de Dios Ruiz-Rosado[1], Luis I. Terrazas[1], Imelda Juárez[1], Lorena Gomez-Garcia[2], Elsa Calleja[1], Griselda Camacho[1], Ana Chávez[1], Miriam Romero[1], Tonathiu Rodriguez[1], Bertha Espinoza[3] and Miriam Rodriguez-Sosa[1]✉

1. Unidad de Biomedicina, Facultad de Estudios Superiores-Iztacala, Universidad Nacional Autónoma de México (UNAM), C. P. 54090, Estado de México, México.
2. Department of Immunology, Instituto Nacional de Cardiología "Ignacio Chávez," México, D.F. 14080 México.
3. Department of Immunology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México. México, D.F. 04510 México.

✉ Corresponding author: Miriam Rodríguez-Sosa, Unidad de Biomedicina, FES-Iztacala, UNAM. Av. de los Barrios # 1, Los Reyes Iztacala, 54090 Tlalnepantla, Edo. de México. Mexico. Phone: (52-55) 5623-1333 ext. 39789. Fax: (52-55) 5623-1225. Email: rodriguezm@campus.iztacala.unam.mx.

© Ivyspring International Publisher. This is an open-access article distributed under the terms of the Creative Commons License (http://creativecommons.org/licenses/by-nc-nd/3.0/). Reproduction is permitted for personal, noncommercial use, provided that the article is in whole, unmodified, and properly cited.

Received: 2014.03.25; Accepted: 2014.07.05; Published: 2014.08.23

Abstract

The C-type lectin receptor mMGL is expressed exclusively by myeloid antigen presenting cells (APC) such as dendritic cells (DC) and macrophages (Mφ), and it mediates binding to glycoproteins carrying terminal galactose and α- or β-N-acetylgalactosamine (Gal/GalNAc) residues. *Trypanosoma cruzi* (*T. cruzi*) expresses large amounts of mucin (TcMUC)-like glycoproteins. Here, we show by lectin-blot that galactose moieties are also expressed on the surface of *T. cruzi*. Male mMGL knockout (-/-) and wild-type (WT) C57BL/6 mice were infected intraperitoneally with 10^4 *T. cruzi* trypomastigotes (Queretaro strain). Following *T. cruzi* infection, mMGL-/- mice developed higher parasitemia and higher mortality rates compared with WT mice. Although hearts from *T. cruzi*-infected WT mice presented few amastigote nests, mMGL-/- mice displayed higher numbers of amastigote nests. Compared with WT, Mφ from mMGL-/- mice had low production of nitric oxide (NO), interleukin (IL)-12 and tumor necrosis factor (TNF)-α in response to soluble *T. cruzi* antigens (TcAg). Interestingly, upon *in vitro T. cruzi* infection, mMGL-/- Mφ expressed lower levels of MHC-II and TLR-4 and harbored higher numbers of parasites, even when mMGL-/- Mφ were previously primed with IFN-γ or LPS/IFN-γ. These data suggest that mMGL plays an important role during *T. cruzi* infection, is required for optimal Mφ activation, and may synergize with TLR-4-induced pathways to produce TNF-α, IL-1β and NO during the early phase of infection.

Key words: mMGL, *Trypanosoma cruzi*, Proinflammatory cytokines, C-Type lectin receptor, Macrophages receptors.

Introduction

Chagas disease, also known as American trypanosomiasis, is a disease caused by the protozoan parasite *Trypanosoma cruzi*. This parasitic infection affects approximately 7-12 million people in Latin America, and 60-80 million more are at risk (1, 2). The natural transmission of the *T. cruzi* parasite to humans and other mammals depends on the triatomine insect belonging to the family (Reduviidae), known as the

kissing bug (3). Inside the host, the parasite is internalized in the cells of the innate immune system. After multiplication by binary fission, the amastigotes are transformed into blood trypomastigotes, which are released into the bloodstream by cell lysis. This stage can infect a wide range of host cells or be taken up by the insect vector through its mouthparts, closing the life cycle (4).

Once an individual has acquired the infection, a progressive disease develops. The acute phase is characterized by a high number of trypomastigotes in the blood as well as, fever and hepatomegaly. The chronic phase presents fewer parasites in the blood. Many patients infected with *T. cruzi* remain asymptomatic. However, 10-20 years after the initial infection, 5-10% of people develop anatomical and functional abnormalities in the esophagus and colon, while approximately 30% develop myocarditis, leading to heart failure or sudden death (4).

The outcome of the immune response depends on the capacity of Antigen Presenting Cells (APCs) to sensing foreign molecular configurations, also known as pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs) expressed on the surface of macrophages (Mφ) or dendritic cells (DC). Activation of PRRs leads to intracellular signals that activate innate immunity and orchestrate the development of an acquired immune response, which is necessary for protection against re-infection (5, 6). Some of these highly specialized receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (6).

In phagocytic cells from both mouse and human, the molecule glycosylphosphatidylinositol (GPI) anchors to *T. cruzi* trypomastigotes, and is recognized as a potent activator of TLR2 (7-10). The glycoinositolphospholipids (GIPLs) and unmethylated CpG motifs on DNA that are present in all stages of the *T. cruzi* life cycle are recognized by TLR4 (11, 12) and TLR9 (13), respectively. For activation of TLR2 and TLR9, the nonsaturated fatty acid chains and periodate-sensitive components from the GPI anchor covalently linked to mucin-like glycoproteins are required to trigger the production of inflammatory cytokines by APCs (11, 14-17).

In contrast to TLRs, CLRs recognize and internalize specific carbohydrates by lectin-glycan interactions. It has been suggested that CLRs work as mediators of microbial recognition and initiators of immune responses (5). The most important molecules from the CLR family include macrophage galactose type C-lectin (MGL), specific for mannose or fucose dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), the mannose receptor (MR), DEC205, and Dectin-1 (18). Some of these receptors may function as adhesion, signaling or antigen-uptake receptors (19-21). Moreover, CLRs have been shown to contribute to the loading of endocytosed antigens on MHC class I or class II, thereby facilitating effective antigen-specific $CD4^+$ and $CD8^+$ T- cell responses (22, 23).

The CLRs are able to trigger distinct signaling pathways that modulate APC functions through the expression of specific molecules and cytokines. In most cases, CLRs promote antigen presentation and determine the polarization of T cells (24-26). However, most evidence about how CLRs shape the immune response and trigger signaling pathways has emerged using viral and bacterial pathogens, fungi or peptides (18). There is no clear evidence that parasites interact with CLRs and activate a specific signaling pathway.

The MGL molecule is a member of the CLR family. Human MGL (hMGL) is a type II C-Type lectin, which is selectively expressed in APCs such as immature dendritic cells (iDC) and Mφ and is overexpressed in subsets of DCs with tolerogenic functions and alternatively activated Mφ. MGL recognizes Gal/GalNAc residues of *N*- and *O*-glycans carried by glycoproteins and /or glycosphingolipids and it promotes endocytosis (27).

It is well-known that endogenous functions for MGLs include pattern recognition of tumor antigens, foreign glycoproteins derived from helminth parasites, and cleaning of apoptotic cell embryos (28). The mechanisms or pathways used for internalizing carbohydrates by MGL have not been fully described, but has been reported that phagocytic cells are able to endocytose antigens through MGL, which them are transported along the endosomal-lysosomal pathway and presented in MHC-II molecule (29). Mice have two homologous copies of hMGL, MGL1 and MGL2, while humans and rats have only one copy (30, 31). Mouse MGL (mMGL) is a transmembrane glycoprotein of 42KD that recognizes Gal/GalNAc and Lewis X and Lewis A structures (30, 32).

In this work, we investigated the role of mMGL in immune response to an acute *T. cruzi* infection using mMGL knockout mice (mMGL-/-). Our results demonstrate that mMGL plays an important role in *T. cruzi* infection and may be useful to prevent *T. cruzi* invasion and to induce inflammatory cytokines and NO production by macrophages.

Materials and Methods

Trypanosoma cruzi lysate antigen. Epimastigotes of *T. cruzi* that had been maintained at 28°C by sequential culture in a liver infusion tryptose medium (LIT) with 25 mg/l of hemin supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U of penicillin/streptomycin were isolated,

kissing bug (3). Inside the host, the parasite is internalized in the cells of the innate immune system. After multiplication by binary fission, the amastigotes are transformed into blood trypomastigotes, which are released into the bloodstream by cell lysis. This stage can infect a wide range of host cells or be taken up by the insect vector through its mouthparts, closing the life cycle (4).

Once an individual has acquired the infection, a progressive disease develops. The acute phase is characterized by a high number of trypomastigotes in the blood as well as, fever and hepatomegaly. The chronic phase presents fewer parasites in the blood. Many patients infected with *T. cruzi* remain asymptomatic. However, 10-20 years after the initial infection, 5-10% of people develop anatomical and functional abnormalities in the esophagus and colon, while approximately 30% develop myocarditis, leading to heart failure or sudden death (4).

The outcome of the immune response depends on the capacity of Antigen Presenting Cells (APCs) to sensing foreign molecular configurations, also known as pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs) expressed on the surface of macrophages (Mφ) or dendritic cells (DC). Activation of PRRs leads to intracellular signals that activate innate immunity and orchestrate the development of an acquired immune response, which is necessary for protection against re-infection (5, 6). Some of these highly specialized receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (6).

In phagocytic cells from both mouse and human, the molecule glycosylphosphatidylinositol (GPI) anchors to *T. cruzi* trypomastigotes, and is recognized as a potent activator of TLR2 (7-10). The glycoinositolphospholipids (GIPLs) and unmethylated CpG motifs on DNA that are present in all stages of the *T. cruzi* life cycle are recognized by TLR4 (11, 12) and TLR9 (13), respectively. For activation of TLR2 and TLR9, the nonsaturated fatty acid chains and periodate-sensitive components from the GPI anchor covalently linked to mucin-like glycoproteins are required to trigger the production of inflammatory cytokines by APCs (11, 14-17).

In contrast to TLRs, CLRs recognize and internalize specific carbohydrates by lectin-glycan interactions. It has been suggested that CLRs work as mediators of microbial recognition and initiators of immune responses (5). The most important molecules from the CLR family include macrophage galactose type C-lectin (MGL), specific for mannose or fucose dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), the mannose receptor (MR), DEC205, and Dectin-1 (18). Some of these receptors may function as adhesion, signaling or antigen-uptake receptors (19-21). Moreover, CLRs have been shown to contribute to the loading of endocytosed antigens on MHC class I or class II, thereby facilitating effective antigen-specific $CD4^+$ and $CD8^+$ T- cell responses (22, 23).

The CLRs are able to trigger distinct signaling pathways that modulate APC functions through the expression of specific molecules and cytokines. In most cases, CLRs promote antigen presentation and determine the polarization of T cells (24-26). However, most evidence about how CLRs shape the immune response and trigger signaling pathways has emerged using viral and bacterial pathogens, fungi or peptides (18). There is no clear evidence that parasites interact with CLRs and activate a specific signaling pathway.

The MGL molecule is a member of the CLR family. Human MGL (hMGL) is a type II C-Type lectin, which is selectively expressed in APCs such as immature dendritic cells (iDC) and Mφ and is overexpressed in subsets of DCs with tolerogenic functions and alternatively activated Mφ. MGL recognizes Gal/GalNAc residues of *N*- and *O*-glycans carried by glycoproteins and /or glycosphingolipids and it promotes endocytosis (27).

It is well-known that endogenous functions for MGLs include pattern recognition of tumor antigens, foreign glycoproteins derived from helminth parasites, and cleaning of apoptotic cell embryos (28). The mechanisms or pathways used for internalizing carbohydrates by MGL have not been fully described, but has been reported that phagocytic cells are able to endocytose antigens through MGL, which them are transported along the endosomal-lysosomal pathway and presented in MHC-II molecule (29). Mice have two homologous copies of hMGL, MGL1 and MGL2, while humans and rats have only one copy (30, 31). Mouse MGL (mMGL) is a transmembrane glycoprotein of 42KD that recognizes Gal/GalNAc and Lewis X and Lewis A structures (30, 32).

In this work, we investigated the role of mMGL in immune response to an acute *T. cruzi* infection using mMGL knockout mice (mMGL-/-). Our results demonstrate that mMGL plays an important role in *T. cruzi* infection and may be useful to prevent *T. cruzi* invasion and to induce inflammatory cytokines and NO production by macrophages.

Materials and Methods

Trypanosoma cruzi lysate antigen. Epimastigotes of *T. cruzi* that had been maintained at 28°C by sequential culture in a liver infusion tryptose medium (LIT) with 25 mg/l of hemin supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U of penicillin/streptomycin were isolated,

washed three times in PBS, and centrifuged at 20,000 g for 15 min (33). Protease inhibitors were added (0.1 to 2 µg/ml apotinin, 0.5 to 2 mM EDTA, 1 to 5 mM phenylmethyl fluoride, 1 µg/ml pepstatin, 50 µg/ml TLCK (a-p-tosyl-L-Lysine chloromethyl Ketone) (Sigma, St. Louis, MO), and parasites were sonicated six times for 10 s each at 50 W using a sonic Dimembrator 300 (Fisher). Parasite destruction was confirmed using a microscope. Parasite lysates were then centrifuged at 20, 000 g for 30 min to separate the soluble fraction, which was stored at -70ºc until use. Total protein content was determined in the soluble fraction (34).

SDS-PAGE and Lectin-Blotting. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and lectin blotting were performed by standard techniques. Briefly, antigen extracts in non-reducing sample buffer were boiled for 5 min at 95°C and separated on 12% polyacrylamide gels at a concentration of 40 µg/well. Separated proteins were transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ, USA) using a Western blotting unit (Bio-Rad). The membrane was blocked overnight at 4°C with 2% (w/v) bovine serum albumin in PBS pH 7.2, washed thoroughly with PBS/Tween 0.1% and incubated with *Artocarpus integrifolia* lectin (Jacalin)-Peroxidase (Sigma-Aldrich), a lectin specific to Gal residues, for 3 h. After washing, bound peroxidase on the membrane was developed with 1: 1000 PBS/ H and diaminobenzidine at a concentration of 2 mg/ ml.

Mice. Eight to ten week-old male C57BL/6 mice were purchased from Harlan (México City, Mexico). The generation of mMGL-deficient mice from C57BL/6 mice by a disruption in mMGL exons 2 and 3 has been previously described (35). mMGL-/- mice on a C57BL/6 background were donated by Glycomics Consortium. All mice were genotyped by PCR. Briefly, total DNA was extracted from the tails of the mice (36), and DNA amplification was performed using 1 µg of DNA, oligo(dT) and specific primers for 35 cycles of 30 sec at 65ºC. Oligos for the *mMGL* gene were (5'-ATGTCATGACTCAGGATC-3' and 5'-CTTGGTCCCAGATCCGTATC-3'), and for the neomicine gene (Neo), 5´-AGGATCTCCTGTCATCT CACCTTGCTCCTG-3' and 3'-AAGAACTCGTCAAG AAGGCGATAGAAGGCG-5´. A PCR fragment of 633 bp to mMGL and 492 bp to Neo were visualized to identify WT or mMGL-/- mice, respectively. Wild type C57BL/6 purchased from Harlan (México) were used as control mice. All animal studies were performed according to the guidelines for the Care and Use of Laboratory Animals, as adopted by the U.S. National Institutes of Health and the Mexican Regulation of Animal Care and maintenance (NOM-062-ZOO-1999, 2001).

Parasites and experimental infections. The Mexican *T. cruzi* TBAR/MX/0000/Queretaro strain belonging to DTU TcI was used in this work. Experimental infections of male mMGL-/- and WT mice were induced by intraperitoneal (i.p.) injection with 10^4 blood trypomastigotes that were obtained from previously infected mice, counted, and suspended in 100 µl of sterile phosphate-buffered saline (PBS). Parasitemia was determined every week by using hemocytometer counts of parasites in the blood diluted 1:10 in PBS with 3.8% sodium citrate.

Histopathology. Non-infected and *T. cruzi* infected hearts from mMGL-/- and WT mice were fixed overnight in formaldehyde and embedded in paraffin blocks, after which 5-µ-thick transverse sections were mounted on slides, stained with hematoxylin and eosin (H&E), and scored as previously described (37). The presence of inflammatory cells was scored as (0) - absent/none, (1) - focal or mild with $\leq$1 foci, (2) - moderate with $\geq$2 inflammatory foci, (3) - extensive with generalized coalescing of inflammatory foci or disseminated inflammation and (4) - severe with diffused inflammation, interstitial edema, and loss of tissue integrity. The foci of pseudocysts (Tc nests) were scored as (0) absent, (1) 0–1 foci, (2) 1–5 foci, and (3) 5 foci. Using an Olympus BX51 microscope (Olympus American, Melville, NY) equipped with a digital video camera, 4 mice per group with 10-slides per mouse were evaluated for each group.

Isolation of macrophages and activation. Peritoneal exudate cells (PECs) were obtained from the peritoneal cavity at 0, 21, 28 and 35 days post *T. cruzi* infection of mMGL-/- and WT mice under sterile conditions using 10 ml of ice-cold Hank´s balanced salt solution (Microlab, México). Following two washes with Hank's solution, red blood cells were lysed by resuspending the cells in Boyle's solution. After two washes, viable cells were counted by trypan blue exclusion (routinely over 95%) with a Neubauer hemocytometer. PECs were adjusted to $5x10^6$ cells/ml in DMEM medium supplemented with 10% fetal calf serum (FCS), 100 U of penicillin/streptomycin, and 2 mM glutamine (all from Gibco-BRL, Grand Island, NY) and were cultured in 24-well plates (Costar, Cambridge, MA, USA). After 2 hours at 37ºC and 5% CO_2, non-adherent cells were removed by washing with warm supplemented DMEM medium. Adherent cells (Mφ) were removed from the plate by washing with 5 mM EDTA in warm PBS and were then readjusted to $1x10^6$ cells/ml. Viability was checked again at this point (>90%), and samples were analyzed by FACS using the macrophage marker F4/80 (Mφ purity was estimated as >85%). One milliliter of Mφ was plated on 12-well plates (Costar), left untreated or

stimulated with LPS (0.5 μg/ml; Escherichia coli 0111:B4 Sigma-Aldrich), or Poly:IC (25 μg/ml; Poly-inosinic-polycytidylic acid potassium salt), or 25 μg/ml of total *T. cruzi* antigen (TcAg). Mφ were incubated for 48 hours at 37°C and 5% CO_2. Supernatants were collected for quantification of cytokine production. Cytokine levels (IL-12, IFN-γ, TNF-α, IL-10, IL-4, and IL-13) were measured using the sandwich ELISA method according to the manufacturer's instructions (Preprotech, México).

Reverse transcriptase-PCR. Total RNA was extracted from Mφ obtained as described above from untreated or *in vitro T. cruzi* infected (21 days after infection) WT or mMGL-/- mice, using the TRIzol reagent (Sigma). cDNA was prepared using a first strand synthesis superscript II kit (Invitrogen) from 5 μg of total RNA. cDNA samples were standardized based on the content of the housekeeping gene Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) cDNA. The primers for GAPDH were F-CTC ATG ACC ACA GTC CAT GC and R-CAC ATT GGG GGT AGG AAC AC (201 bp). The primers for Arginase-1 were F-CAG AAG AAT GGA AGA GTC AG and R-CAG ATA TGC AGG GAG TCA CC (250 bp). The primers for Ym1 were F-TCA CAG GTC TGG CAA TTC TTC TG and R-TTT GTC CTT AGG AGG GCT TCC TC (436 bp). The primers for TNF-α were F-GGC AGG TCT ACT TTG GAG TCA TTG C and R-ACA TTC GAG GCT CCA GTG AAT TCG (307 bp). The primers for inducible nitric oxide synthase were F-CTG GAG GAG CTC CTG CCT CAT G and R-GCA GCA TCC CCT CTG ATG GTG (449 bp). The primers for IL-1β were F-GAG TGT GGA TCC CAA GCA AT and R-CTC AGT GCA GGC TAT GAC CA (500 bp). The primers for TLR-4 were ACC TGG CTG GTT TAC ACG TC and R-CTG CCA GAG ACA TTG CAG AA (201 bp). The primers for TLR-3 F-CCC CCT TTG AAC TCC TCT TC and R-TTT CGG CTT CTT TTG ATG CT. The primers for TLR-2 F-AAG AGG AAG CCC AAG AAA GC and R-CGA TGG AAT CGA TGA TGT TG (199 bp). Polymerase chain reaction (PCR) was performed in a total volume of 50 μL in PCR buffer in the presence of 10 mM dNTPs, 15 pM each primer, and 1.5 U of kappa TaqDNA polymerase (kapabiosystems Boston M.A, USA) using a XP-cycler (Bioer, Switzerland). After 35 cycles of amplification, the PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

***In vitro T. cruzi* infection and flow cytometry analysis of macrophages**. One milliliter ($1x10^6$) of Mφ obtained as described above from WT or mMGL-/- mice was plated, and infected *in vitro* with epimastigores of *T. cruzi* (ratio 1: 10) for two hours. Following infection, parasites were washed off. Mφ were incubated at 4°C for 15 minutes in blocking buffer and 2% FCS, 10 μg/ml anti-CD16/32 in FACS buffer (PBS supplemented with 2mM EDTA and 0.5% BSA), followed by staining for 20 minutes on ice with the antibodies (Ab) of interest at the appropriate dilution as determined by titration. Abs included APC-conjugated anti-F4/80, FITC-conjugated MHC-II and PE-conjugated TLR-4 as well as appropriate isotype control Abs (all Abs from Biolegend, San Diego, CA, USA).

For infection analysis; $1x10^6$ Mφ obtained from WT or mMGL-/- mice were plated, and infected *in vitro* with epimastigores of *T. cruzi* (ratio 1: 10) for two hours. For infection analysis, epimastigotes of *T. cruzi* were washed in PBS and resuspended at $1x10^7$/ml in 5 mM carboxyfluorescein succinimidyl ester (CFSE) in serum-free DMEM for 15 min at 37oC. Following infection, parasites were washed off, and Mφ infected and not infected were incubated in DMEM medium supplemented with 10% FCS for 2, 6, 12 or 24 hours at 37oC and 5% CO2.

For infection analysis on activated Mφ, $1x10^6$ thioglycollate-elicited Mφ were left untreated or treated for 24 h with IFN-γ or IFN-γ/LPS. Cells were washed and infected with CFSE labeled epimastigotes of *T. cruzi* for 2 h (10:1 parasite to Mφ ratio), after this the parasites were washed off.

All cells described in this section were washed 3x in FACS buffer and fixed in 0.8% paraformaldehyde before acquisition and analysis (BD FACStation and FlowJo software).

Statistical analysis. Comparisons between WT and mMGL-/- groups were made by using Student's unpaired *t* test. P values of ‹ 0.05 were considered significant. For survival assays, a log-rank test was used with the Graph Pad computer program (Graph Pad 6, San Diego, CA).

Results

Intact carbohydrates on total antigens of *T. cruzi* are rich in glycoproteins bearing galactose residues. Given that *T. cruzi* parasites are rich in carbohydrates (38), we developed a PAGE (Fig. 1A) and lectin-blot analysis (Fig. 1B) to determine whether the *T. cruzi* Queretaro strain contained glycoproteins bearing terminal Gal and GalNAc sugars, which are recognized by mMGL with high affinity. According to the lectin-blot analysis (Fig. 1B) the total soluble antigens of *T. cruzi* were highly recognized by Jacalin lectin, indicating the presence of Gal residues, similar to other reports with different *T. cruzi* strains (39).

mMGL-/- mice develop high parasitemia levels and increased mortality to *T. cruzi* infection. To investigate the role of mMGL in immunity to *T. cruzi*, mMGL-/- and WT mice were i.p. infected with 10^4

metacyclic trypomastigotes of *T. cruzi,* and parasitemia was monitored weekly. *T. cruzi*-infected mMGL-/- mice developed significantly higher blood parasitemia levels than similarly infected WT mice, which contained nearly four-fold fewer parasites in their blood by days 21 to 35 post infection (pi) (Fig. 2A, P <0.05). Both groups developed blood parasitemia on day 14 pi, but on day 28 pi, mMGL-/- mice displayed significantly greater levels of parasitemia, which peaked at day 28 pi and was positively detected until day 42 pi (Fig. 2A). In contrast, the maximum peak of parasitemia in WT mice was observed on day 21 pi, and these mice controlled the infection by day 42. Furthermore, mMGL-/- mice succumbed to *T. cruzi* infection as early as day 23 pi, and only 37% of them survived to day 42, whereas 80% of the WT mice survived throughout the infection (Fig. 2B).

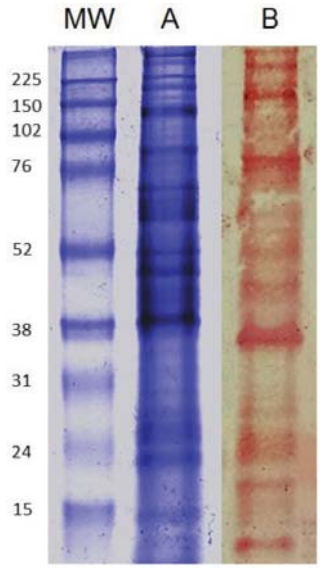


Figure 1. Total *T. cruzi* antigen is rich in glycoproteins bearing galactose (Gal) and N-acetylgalactosamine (GALNAc). TcAg were separated by SDS-PAGE and transferred to NC sheets that were used to detect N-linked glycans with horseradish peroxidase (HRP)-conjugated Jacalin. *T. cruzi* antigen lectin blood, SDS-page A); Jacalin-Blot B). Antigens were from different infected mice. MW indicates the molecular weight markers in kilodaltons (kDa).

***T. cruzi*-infected mMGL-/- mice develop severe heart pathology.** The heart is one of the main target organs affected by *T. cruzi* infection, therefore we evaluated whether the higher mortality of mMGL-/- mice was associated with heart damage. We found that on day 21 pi, the hearts of WT mice showed low tissue parasitism with moderate inflammatory mononuclear cell infiltration and no major histopathological signs of lesions (Fig. 3A at 21 and 28 days post infection -dpi and 3B). In contrast, heart histopathology sections from infected mMGL-/- mice showed an intense inflammatory reaction (Fig. 3A at 21 and 28 dpi and 3B), with a marked increase in amastigote nests (Fig. 3C) accompanied by severe heart injury due to large necrotic lesions. These results suggest that mMGL-/- mice succumb to the classical pathophysiology of the infection, which is typical of the severe acute phase of experimental Chagas' disease.

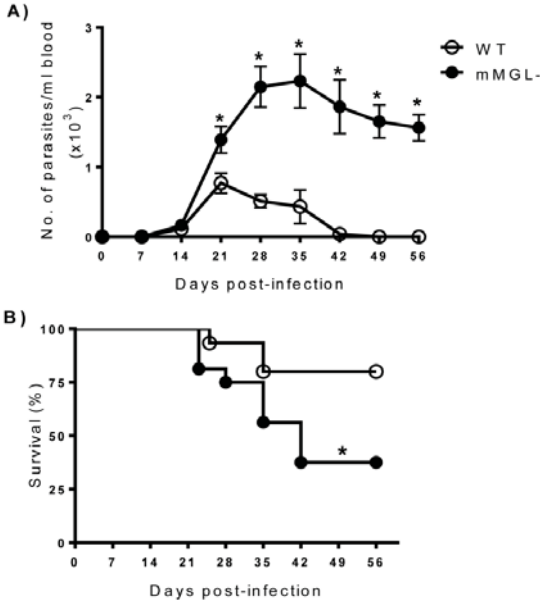


Figure 2. mMGL-/- mice succumb to acute *T. cruzi* infection. WT and mMGL-/- mice were infected with 10^4 *T. cruzi* parasites. Parasitemia A), data shown represent the mean ±SE of at least 21 mice per data point corresponding to three independent experiments (7 mice per experiment). Survival rate B), data shown represent the mean ±SE of 15 mice corresponding to three independent experiments (5 mice per experiment). Both experimental groups were monitored every week until seven weeks p.i. *P<0.05 with respect to WT. Student's *t* test, and log rank test for parasite load and survival were used respectively (Graph Pad Prism 6).

mMGL-/- Mφ from *T. cruzi* infected-mice have impaired pro-inflammatory cytokine expression. Given that the inflammatory immune response is a well-defined mechanism to control early *T. cruzi* dissemination (16, 40), we decided investigate the pro-inflammatory profile of Mφ obtained from mMGL-/- and WT mice during acute *T. cruzi* infection. Peritoneal Mφ from uninfected and *T. cruzi* infected mice at 21 and 35 dpi were left untreated or treated for 48 hours with LPS, POLY:IC or TcAg, and supernatants were recovered for cytokine detection by ELISA. As expected, uninfected Mφ (day 0) from mMGL-/- or WT mice showed similar levels of NO, TNF-α, IL-12 and IL-10 in response to all stimuli (Fig. 4A-D). As the infection progressed, mMGL-/- Mφ left untreated exhibited lower levels of NO at 21 and 35 dpi (Fig. 4A) and lower levels of TNF-α, IL-12 and

IL-10 at day 21 post-infection, compared with their similar WT Mφ (Figure 4A).

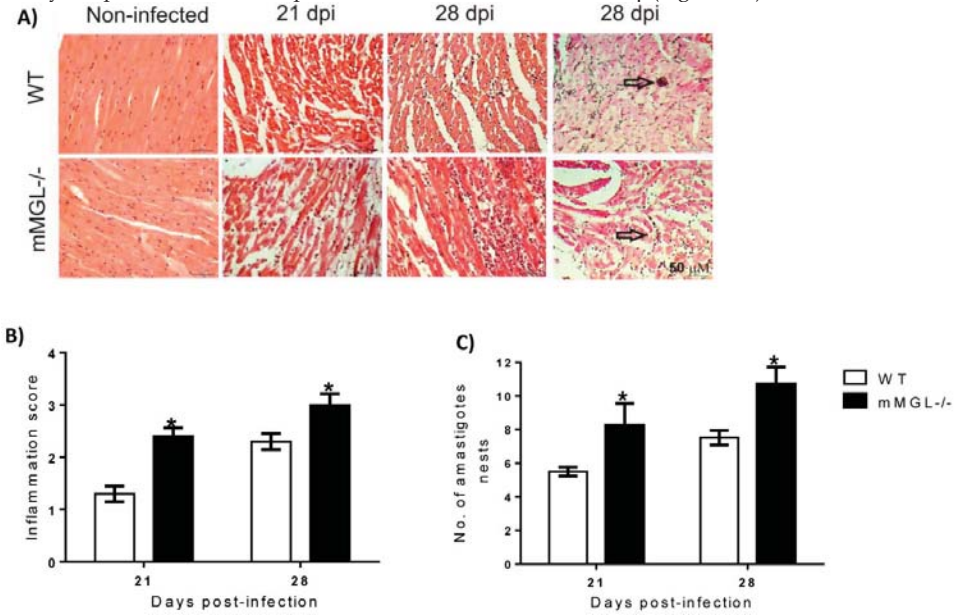


Figure 3. Hearts from mMGL-/- mice display higher *T. cruzi* parasitism and inflammation compared with WT mice. Histology of hearts post-*T. cruzi* infection A). Quantitative scoring of heart inflammation from H&E-stained tissue sections B). Number of amastigote nests in 25 histopathological fields C). Representative H&E images of heart tissue sections shown myocyte fibers cut in longitudinal (blue: nuclear, pink: muscle/cytoplasm/keratin) (arrows indicate parasite nests) (magnification: 40X, Olympus BX51 microscope). Data are expressed as the means ±SE of the means (*n* 4 to 5 animals per group). Mice were killed at 0, 21 and 28 days p.i. Arrows point to parasite nests. *P≤ 0.05 with respect to WT values obtained the same day. Student's *t* test was used (Graph Pad Prism 6).

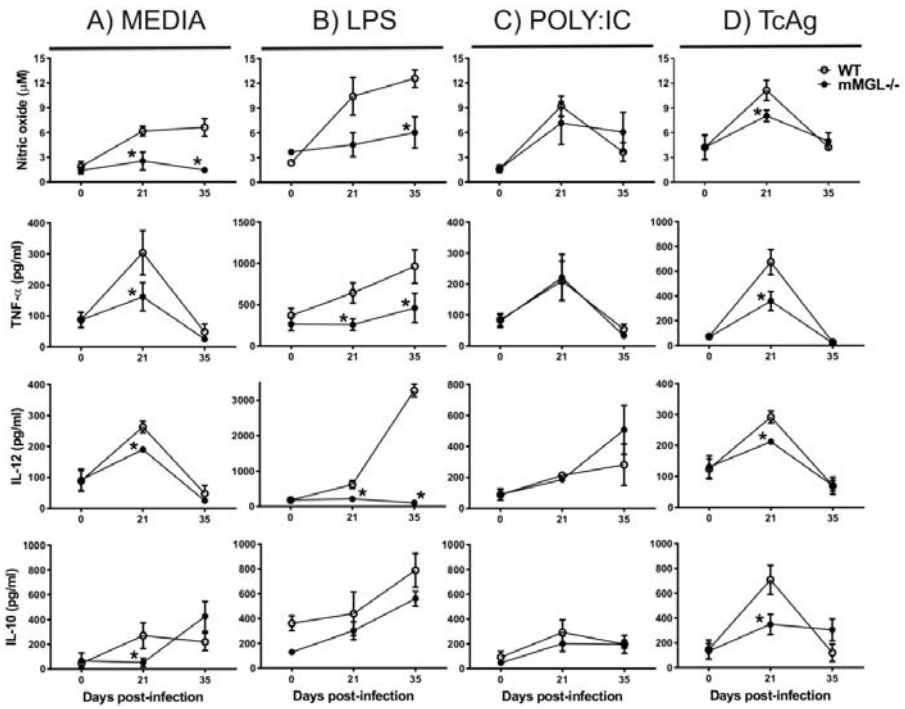


Figure 4. NO_2-, TNF-α, and IL-12 production are reduced in response to LPS or TcAg in mMGL-/- Mφ compared with WT Mφ. Naïve or infected Mφ from WT or mMGL-/- mice were recovered after 21, 28 and 35 days post *T. cruzi* infection. Mφ were left untreated or treated for 48 hours with LPS (0.5 μg/ml), POLY: IC (25 μg/ml) or TcAg (25 μg/ml) as indicated.

Supernatants were recovered and the levels of IL-12 A), TNF-α B), IFN-γ C) were measured by ELISA sandwich. Results are shown as the means of replicate samples +/- SEM and are representative of three experiments. *P<0.05 by Student's *t* test (GraphPad Prism 6).

LPS is considered a Th1 activating signal that favors inflammatory cytokine production by Mφ (41). Here, we show that in response to LPS, mMGL-/- Mφ from 21 or 35 days after infection displayed impaired production of NO, TNF-α and IL-12 but not IL-10 compared with WT Mφ (Fig. 4B). In response to TcAg, mMGL-/- Mφ produced lower levels of NO, IL-12 and IL-10 only at 21 dpi (Fig. 4D), whereas no differences were observed with POLY:IC stimulation (Fig. 4C). These results indicate that mMGL-/- Mφ coming from *T. cruzi* infected animals may less activated, or may display an alternative activation Mφ (AAMφ) phenotype.

mMGL-/- Mφ from 21 days post-*T. cruzi* infection display a defect in their activation more than a phenotype switch. Signals encountered by developing Mφ during migration determine the development of highly divergent Mφ phenotypes with specific functional properties at the site of inflammation or infection (42). Classically activated (CA)Mφ, activated by Th1-type signals such as IFN-γ, produce high levels of nitric oxide (NO), enhancing antimicrobial and cytotoxic properties (43, 44). AAMφ are dependent on the products of activated Th2 cells, such as IL-4 and IL-13, and play important roles in allergy and the response to parasitic infection (45, 46). AAMφ express secretory lectin Ym-1 and Arginase-1 (Arg-1) over NO. Recently, mMGL has also been considered a marker for AAMφ elicited on Mφ and DCs during infection with *Trypanosoma brucei* or *Taenia crassiceps* (47, 48). In contrast, CAMφ produce high levels of NO from inducible nitric oxide synthase (iNOS), and they do not express Ym1 and Arg-1(41).

In order to determine whether the impaired inflammatory cytokine production in mMGL-/- Mφ was due to a phenotype switch from CAMφ to AAMφ, we analyzed the gene expression of Arg-1, Ym-1 and iNOS as Mφ phenotype markers, TNF-α and IL-1β as inflammatory markers, and TLR-2, TLR-3 and TLR-4 as activation markers. Peritoneal Mφ recovered from mMGL-/- and WT mice 21 days after infection and Mφ from non-infected mice were left untreated (media) or treated overnight with LPS, POLY:IC or TcAg for gene expression analysis by RT-PCR. We found that non-infected mMGL-/- Mφ showed high Arg-1, Ym-1 and TLR3 expression in response to LPS (Fig. 5 B, C and H). High Arg-1 and TLR3 expression was observed in response to TcAg (Fig. 5 B, H), but similar mRNA levels of TNF-α, iNOS, IL-1β, TLR-4 and TLR-2 in response to LPS compared with WT-Mφ (Fig. 5 D, E, F, G and I). No or low mRNA expression of Ym-1, IL-1β and TLR-4 was observed in response to TcAg compared with WT-Mφ (Fig. 5 C, F and G).

Untreated (media) mMGL-/- Mφ from 21 dpi showed similar expression of Arg-1, Ym-1, IL-1β, TLR3 and TLR2 (Fig. 5 B, C, F, H, and I -media) but low expression of iNOS, TNF-α and TLR-4 in these basal condition (Fig 5 D, E, G -media). In response to LPS, 21 day post-infection Mφ showed similar mRNA expression of Arg-1 and Ym-1 (Fig 5B, C) but low mRNA expression of iNOS, TNF-α, IL-1β and TLR-4 (Fig. 5 D, E, F, G). In response to POLY:IC, low mRNA expression of Arg-1, iNOS and TNF-α was observed (Fig. 5B, E, D, F and G -21 dpi). In response to TcAg, mMGL-/- Mφ showed high Ym-1 expression (Fig. 5C -21 dpi), similar levels of iNOS, TLR3 and TLR-2 (Fig. 5E, H and I -21 dpi), and no or low levels of Arg-1, TNF-α, IL-1β and TLR-4 compared with WT Mφ (Fig. 5B, D, F, and G. -21 dpi).

mMGL-/- Mφ display deficiencies in MHC-II and TLR-4 cell surface activation markers during *in vitro* infection with *T. cruzi*. In order to rule out the possibility that altered activation status could be present in mMGL-/- Mφ, we studied the surface expression of MHC class II (MHC-II), co-stimulatory molecules CD80 (B7.1), CD86 (B7.2) and TLR-4. Peritoneal naïve Mφ from WT or mMGL-/- mice were infected *in vitro* with trypomastigotes of *T. cruzi* at a ratio of 1:10 and marker expression was monitored in uninfected Mφ (0 hpi) and *T. cruzi*-infected Mφ at 2, 6, 12 and 24 hpi. Uninfected mMGL-/- Mφ expressed similar levels of MHC-II (Fig. 6A, 0 hpi), CD80 (data not shown), CD86 (data not-shown) and TLR-4 (Fig. 6B, 0 hpi).

Upon *T. cruzi* infection, the percentage of all markers was increased in both mMGL-/- and WT-Mφ from two hours until 24 hpi. Similar expression levels of CD80 and CD86 were observed, but significantly less expression of MHC-II and TLR-4 was observed on mMGL-/- Mφ at 2, 6, 12 and 24 hpi compared with WT Mφ (Fig. 6 A and B, respectively).

Next, we asked whether on the altered activation of mMGL-/- Mφ could reflect their ability to control *T. cruzi* infection. To address this question, *T. cruzi* epimastigotes were labeled with CFSE and used to infect Mφ from healthy mMGL-/- and WT mice *in vitro*, then Mφ were recovered and analyzed by flow cytometry. This allowed the comparison of parasite uptake between mMGL-/- and WT Mφ. Interestingly mMGL-/- Mφ harbored significantly more parasites after 2, 6, 12 and 24 hours post-infection (hpi) (Fig. 7 A) than their WT counterparts, which suggested that the presence of mMGL is important in delaying the entry of parasites into the host cell.

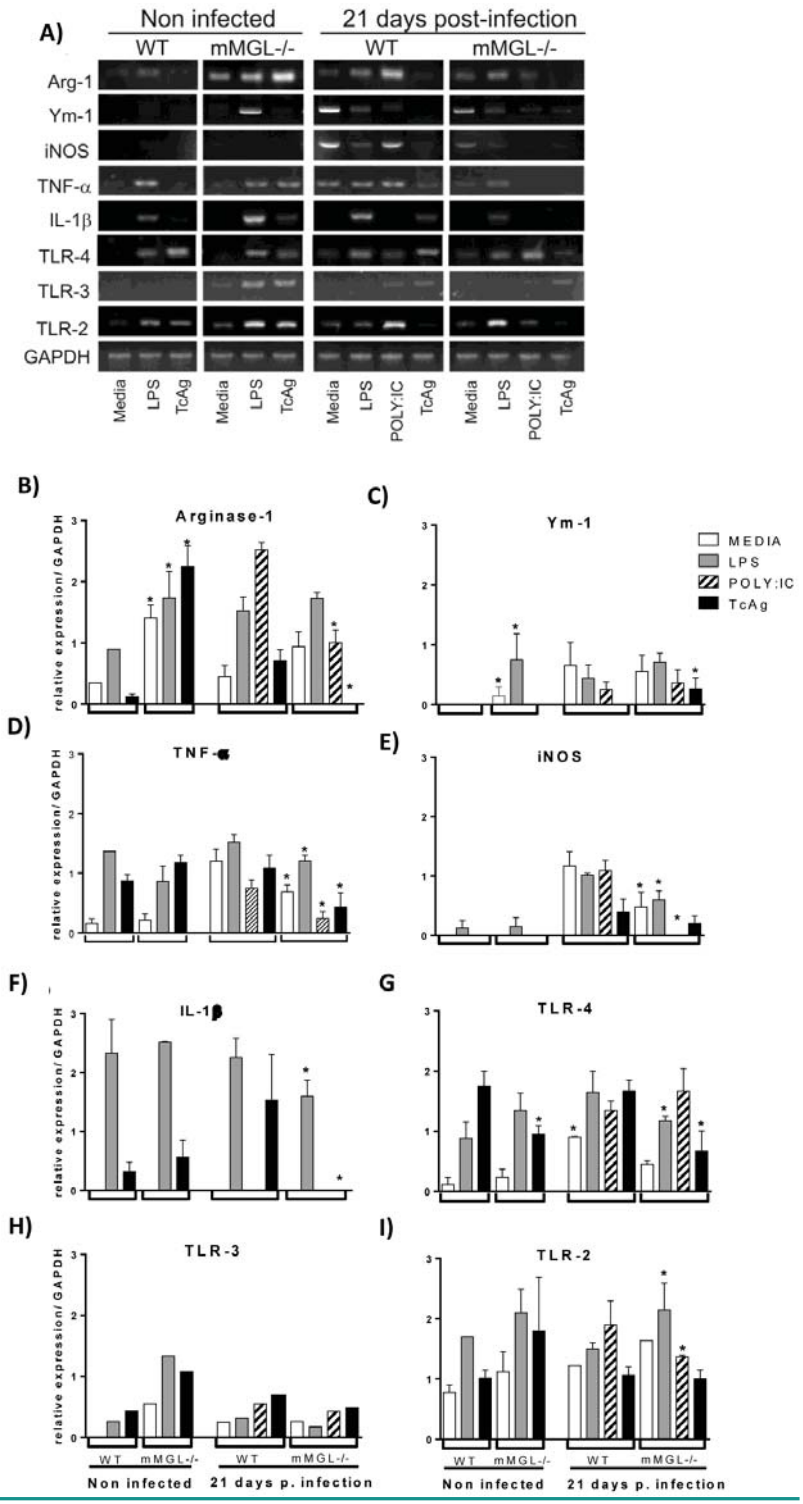


Figure 5. Mφ Phenotype from WT and mMGL-/- 21 days post-*T. cruzi* infection. Naïve or infected Mφ from WT or mMGL-/- mice were recovered 21 days after *T. cruzi* infection. Mφ were left untreated or treated for 24 hours with LPS (5 μg/ml), POLY: IC (25 μg/ml) or TcAg (25 μg/ml), as indicated. The cells were recovered for RNA expression analysis A) of Arginase1 B), Ym1 C), TNF-α D), iNOS E), IL-1β F), TLR-4 G), TLR-3 H) and TLR-2 I) by RT-PCR. Results are shown as the mean of triplicate samples (+/- S.E.M.) and are representative of three independent experiments. *P<0.05 by Student's *t* test (GraphPad Prism 6).

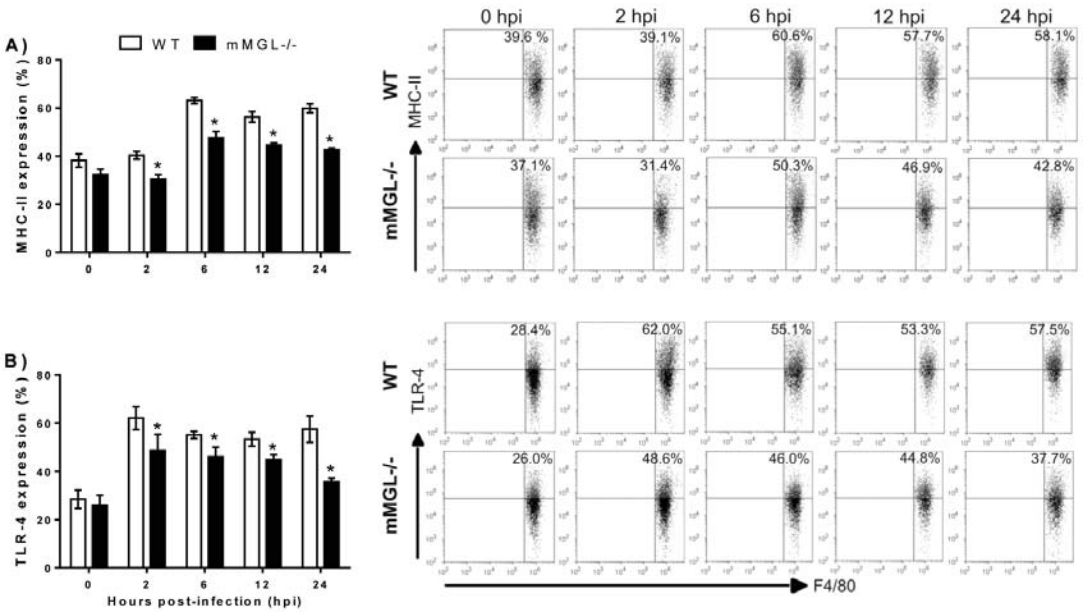


Figure 6. Effect of *T. cruzi* infection on the cell surface activation markers of WT and mMGL-/- Mφ. Naïve Mφ from WT or mMGL-/- mice were infected *in vitro* with trypomastigotes of *T. cruzi* at 10:1parasite to Mφ ratio. The cells were recovered and double-stained for F4/80 and MHC-II A), and TLR-4 B). Flow cytometry graphs show dot plots of F4/80-gated Mφ. Results are representative of three experiments. *P<0.05 by Student's *t* test (GraphPad Prism 6).

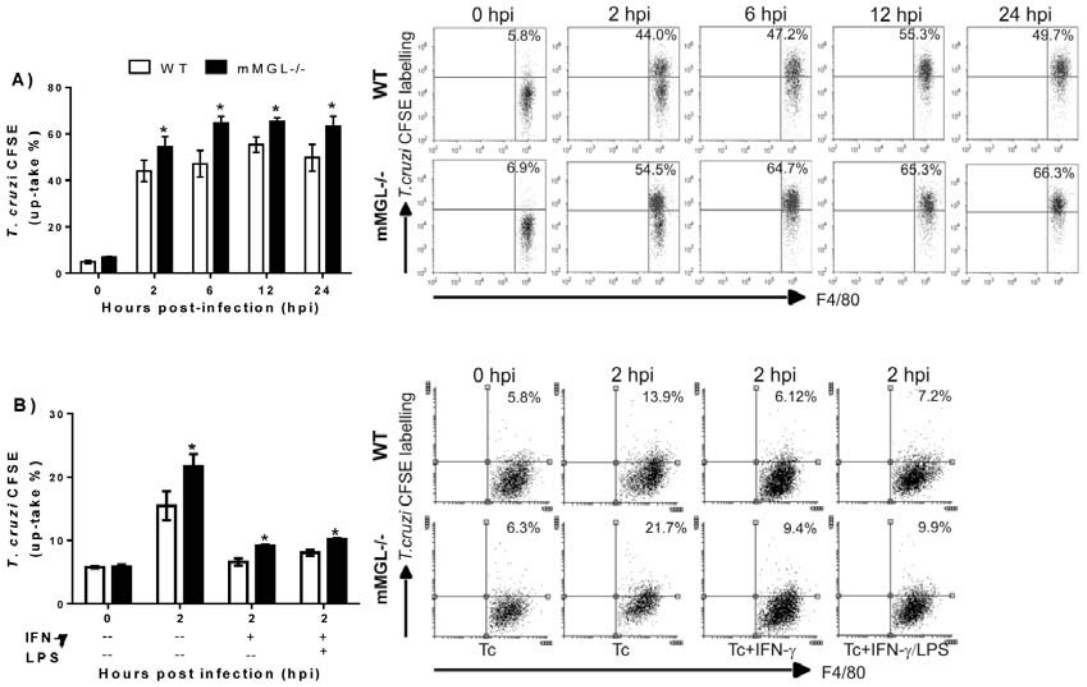


Figure 7. Untreated, IFN-γ and LPS/IFN-γ primed mMGL-/- Mφ take up most *Trypanosoma cruzi* parasites. Peritoneal derived WT or mMGL-/- Mφ were untreated A) or treated 24 h with IFN-γ or LPS/IFN-γ B), followed by infection with *T. cruzi* at 10:1 parasite to Mφ ratio. *T. cruzi*-CFSE-labelling that were not taken up were washed off 2 hours post infection, and parasite uptake determined by flow cytometry at 0, 2, 6 and 12 hours post infection A), or 0 and 2 hours post infection B). Flow cytometry graphs show dot plots of F4/80-gated Mφ. Results are representative of three experiments to A) and two experiments to B). *P<0.05 by Student's t test (GraphPad Prism 6).

To investigate whether the Th1 activating signals, like IFN-γ or IFN-γ/LPS could alter the mMGL-/- Mφ activation. Thioglycollate-elicited Mφ mMGL-/- and WT were left untreated o treated 24 h with IFN-γ or LPS/IFN-γ, infected with CFSE labeled epimastigotes of *T. cruzi* and analyzed by cytometry. As expected, both stimulus IFN-γ and LPS/ IFN-γ act to reduce the parasite uptake on WT as mMGL-/- Mφ. However, this effect was significantly lesser on mMGL-/- Mφ, which showed higher number of parasites compared with WT Mφ (Fig. 7 B). This result demonstrates that mMGL is important to IFN-γ or LPS/IFN-γ stimulus works to activate efficiently Mφ.

Discussion

New evidence has recently emerged about the role of CLRs in modulating the activation and function of APC (18, 49, 50). Some of these studies suggest that MGL improves DC antigen presentation and triggers distinct signaling pathways that regulate APC functions through the expression of specific molecules and cytokines that modulate the innate and adaptive immune response (24, 51). This hypothesis has been driven by the knowledge that MGL works as a molecular target for Gal/GalNAc or Tn-carrying tumor-associated antigens on DCs (24, 25, 52) and is supported by subsequent data showing that mMGL recognizes Gal/GlalNAc residues carried by glycoproteins and/or glycosphingolipids present on the helminth *Schistosoma mansoni* inducing phagocytosis, endocytosis, enhancing parasite glycopeptide presentation and promoting the polarization to Th2 responses (27, 53). Despite evidence pointing to a role for MGL in the immune response to these parasites, the function of MGL in the host defense against protozoa or other helminth parasites rich in Gal/GalNAc residues has not been determined. Therefore, in this study, we evaluated the *in vivo* role of mMGL in *T. cruzi* infection using MGL-/- and WT mice.

First, we demonstrated by Jacalin-Peroxidase lectin blotting that a pathogenic *T. cruzi* strain (Queretaro) has Gal residues on its membrane antigens that can be recognized by mMGL receptors on APCs. This observation is in line with previous reports from other *T. cruzi* strains which demonstrated that this parasite displays highly glycosylated membrane proteins related to the invasion of host cells through recognition by CLRs (31, 54), mainly DEC205 and SIGN (18, 55). However, the role of CLRs *in vivo* has not been determined. Here, we used mMGL-/- mice to demonstrate for the first time that the CLR mMGL plays a critical role in the recognition of and resistance to a protozoan infection. We showed that mMGL-/- mice are highly susceptible to experimental *T. cruzi* infection, harboring prolonged and higher parasitemia levels, severe cardiac immunopathology and increased mortality. These findings indicate that mMGL plays a major role in the host defense against acute *T. cruzi* infection.

Mφ, specifically CAMφ, mediate microbial destruction and play an important role in controlling parasite replication during the acute phase of *T. cruzi* infection by enhancing their microbicidal activity by increasing NO and the proinflammatory cytokines IL-12, TNF-α and MIF (56, 57). We showed that the peritoneal Mφ population generated at 21 days after *T. cruzi* infection in mMGL-/- mice produced significantly less NO, TNF-α, IL-12 and IL-10. Moreover, this Mφ population did not respond as expected to *ex vivo* proinflammatory stimuli (LPS) or to TcAg, but they did respond efficiently to POLY:IC. The lack of proinflammatory cytokines and NO production in early infection in response to *ex vivo* stimuli with LPS or TcAg in mMGL-/- Mφ could be explained by the following: 1) The development of different Mφ phenotypes in mMGL-/- compared with WT infected *T. cruzi* mice. However, this possibility was discarded because we did not observe a phenotype switch in mMGL-/- Mφ. 2) Inadequate Mφ activation that could be dependent on a surface receptor because with POLY:IC stimuli, a ligand to intracellular TLR-3, the mMGL-/- Mφ produced similar levels of cytokines compared with WT Mφ. Thus, mMGL may be required for the efficient production of NO, TNF-α, IL-12 and IL-10 in innate immunity against *T. cruzi* infection.

These observations suggest there was no clear phenotype switch in mMGL-/- Mφ toward an AAM profile. However, our data may reflect a less mature activation state in mMGL-/- Mφ, independently of whether the Mφ come from *T. cruzi* infected mice, which suggests that mMGL-/- Mφ may require signaling through a pathogen recognition receptor such as mMGL before becoming responsive to LPS or TcAg.

The current model states that ligand binding to CLRs elicits signaling cascades that modulate immune responses. Some CLRs, such as Dectin-1 and CLEC9A/DNGR-1, clearly promote immunity through ITAM-like motifs within their cytoplasmic tails, leading to the production of several inflammatory cytokines (49). Strikingly, DC-SIGN and DCIR do not seem to act individually, as their signaling pathways require co-triggering of a TLR molecule for their effects to become apparent. Also, MGL induces IL-10 secretion after antibody crosslinking stimulation (58) and has been reported to trigger the phosphorylation of extracellular signal-regulated kinase 1, 2 (ERK1, 2) and nuclear factor-κB activation. However, this finding has not been tested with "natural ligands" but

with strong activation such as direct antibody stimulation.

On the other hand, MGL engagement improved DC performance as antigen-presenting cells, promoting the up-regulation of maturation markers (HLAII-DR, CD83, CD86, CD40), enhancing motility, and increasing antigen-specific CD8+ activation (25). Recently, the capacity of MGL to modulate TLR-2 signaling has been reported (51). In accordance with this, we showed that upon *in vitro T. cruzi* infection, mMGL-/- Mφ displayed significantly less expression of MHC-II and TLR-4. This is consistent with previous studies showing that MCH-II and TLR-4 expression is essential to develop "activated" Mφ with the ability to kill *T. cruzi* parasites (10). Because TLR4-mediated responses to *T. cruzi* have been reported as one of the main pathways for inducing early cytokine production (12), the low expression levels of TLR-4 on mMGL-/- Mφ may explain the low proinflammatory cytokine production by these cells. It is possible that mMGL engagement may couple to TLR-4 signal transduction for increased TNF-α and IL-1β secretion by Mφ during *T. cruzi* infection. Consistent with this, mMGL-/- Mφ were infected more than WT Mφ. This defect on mMGL-/- Mφ was persistent even when mMGL-/- Mφ were previously primed with IFN-γ or LPS/IFN-γ. The ability of WT Mφ under IFN-γ or IFN-γ/LPS stimulation are able to control parasite number more effectively than mMGL-/- Mφ may be explained by signals that WT Mφ encounter with *T. cruzi* or IFN-γ/LPS causing the upregulation of TLR4. Interestingly, no changes were observed in CD80, CD86, TLR-3 and TLR-2 expression, which suggests that the role of mMGL in upregulating costimulatory molecules may depend on the type of triggering ligand and the time of exposure.

These results, together with the finding that mMGL-/- mice were more susceptible to *T. cruzi* infection, suggest that mMGL is required for optimal Mφ activation and may synergize with TLR-4-induced pathways to produce TNF-α, IL-1β and NO during the early phase of *T. cruzi* infection. These observations contribute to the understanding of the inflammatory properties of the mMGL molecule, pointing to its potential role as an important modulator of the immune response during *T. cruzi* infection, and perhaps in other parasitic diseases. Moreover, mMGL may act as a CLR that plays a critical role in determining the quality of the adaptive immune response to this parasite. A better characterization of the effects of mMGL on APCs involved in innate and adaptive immunity in response to parasitic diseases is therefore of great interest.

Acknowledgments

The authors would like to thank MVZ Leticia Flores for the excellent care of the animals and Thalia Pacheco and Pablo F. Moreno for assistance with the histology pictures. We also thank the awards program for academic staff (PASPA-UNAM-2013) for partially supporting the academic exchange with Ohio State University, USA for MRS, and CONACYT-México for supporting a PhD fellowship for the first author to obtain her degree in Biomedical Sciences, UNAM. This work was supported by grant 152224 from CONACYT, 2012-23 from PAPCA-FESI 2011-12, and IN212412 from UNAM-DGAPA-PAPIIT.

Conflict of Interests

The authors declare that there is no conflict of interests.

References

1. Carabarin-Lima A, Gonzalez-Vazquez MC, Rodriguez-Morales O, Baylon-Pacheco L, Rosales-Encina JL, Reyes-Lopez PA, et al. Chagas disease (American trypanosomiasis) in Mexico: an update. Acta Trop. 2013;127(2):126-35.
2. Barrias ES, de Carvalho TM, De Souza W. Trypanosoma cruzi: Entry into Mammalian Host Cells and Parasitophorous Vacuole Formation. Front Immunol. 2013;4:186.
3. Teixeira SM, Vieira LQ, Gazzinelli RT. Genomics, pathogenesis and control of infection with protozoan parasites. Trends Parasitol. 2002;18(2):52-4.
4. Junqueira C, Caetano B, Bartholomeu DC, Melo MB, Ropert C, Rodrigues MM, et al. The endless race between Trypanosoma cruzi and host immunity: lessons for and beyond Chagas disease. Expert Rev Mol Med; 2010;12:e29.
5. Davicino RC, Elicabe RJ, Di Genaro MS, Rabinovich GA. Coupling pathogen recognition to innate immunity through glycan-dependent mechanisms. Int Immunopharmacol.
6. Gazzinelli RT, Denkers EY. Protozoan encounters with Toll-like receptor signalling pathways: implications for host parasitism. Nat Rev Immunol. 2006;6(12):895-906.
7. Camargo MM, Andrade AC, Almeida IC, Travassos LR, Gazzinelli RT. Glycoconjugates isolated from Trypanosoma cruzi but not from Leishmania species membranes trigger nitric oxide synthesis as well as microbicidal activity in IFN-gamma-primed macrophages. J Immunol. 1997;159(12):6131-9.
8. Camargo MM, Almeida IC, Pereira ME, Ferguson MA, Travassos LR, Gazzinelli RT. Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from Trypanosoma cruzi trypomastigotes initiate the synthesis of proinflammatory cytokines by macrophages. J Immunol. 1997;158(12):5890-901.
9. Talvani A, Ribeiro CS, Aliberti JC, Michailowsky V, Santos PV, Murta SM, et al. Kinetics of cytokine gene expression in experimental chagasic cardiomyopathy: tissue parasitism and endogenous IFN-gamma as important determinants of chemokine mRNA expression during infection with Trypanosoma cruzi. Microbes Infect. 2000;2(8):851-66.
10. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001;167(1):416-23.
11. Oliveira AC, Peixoto JR, de Arruda LB, Campos MA, Gazzinelli RT, Golenbock DT, et al. Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi. J Immunol. 2004;173(9):5688-96.
12. Medeiros MM, Peixoto JR, Oliveira AC, Cardilo-Reis L, Koatz VL, Van Kaer L, et al. Toll-like receptor 4 (TLR4)-dependent proinflammatory and immunomodulatory properties of the glycoinositolphospholipid (GIPL) from Trypanosoma cruzi. J Leukoc Biol. 2007;82(3):488-96.
13. Shoda LK, Kegerreis KA, Suarez CE, Roditi I, Corral RS, Bertot GM, et al. DNA from protozoan parasites Babesia bovis, Trypanosoma cruzi, and T. brucei is mitogenic for B lymphocytes and stimulates macrophage expression of interleukin-12, tumor necrosis factor alpha, and nitric oxide. Infect Immun. 2001;69(4):2162-71.
14. Campos MA, Gazzinelli RT. Trypanosoma cruzi and its components as exogenous mediators of inflammation recognized through Toll-like receptors. Mediators Inflamm. 2004;13(3):139-43.
15. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. J Immunol. 2006;177(6):3515-9.

16. Campos MA, Closel M, Valente EP, Cardoso JE, Akira S, Alvarez-Leite JI, et al. Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88. J Immunol. 2004;172(3):1711-8.
17. Ropert C, Gazzinelli RT. Regulatory role of Toll-like receptor 2 during infection with Trypanosoma cruzi. J Endotoxin Res. 2004;10(6):425-30.
18. Vazquez-Mendoza A, Carrero JC, Rodriguez-Sosa M. Parasitic infections: a role for C-type lectins receptors. Biomed Res Int. 2013;2013:456352.
19. Engering A, Geijtenbeek TB, van Vliet SJ, Wijers M, van Liempt E, Demaurex N, et al. The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells. J Immunol. 2002;168(5):2118-26.
20. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med. 2003;197(9):1119-24.
21. Chakraborty P, Ghosh D, Basu MK. Modulation of macrophage mannose receptor affects the uptake of virulent and avirulent Leishmania donovani promastigotes. J Parasitol. 2001;87(5):1023-7.
22. Svajger U, Anderluh M, Jeras M, Obermajer N. C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. Cell Signal. 2010;22(10):1397-405.
23. Lloyd DH, Viac J, Werling D, Reme CA, Gatto H. Role of sugars in surface microbe-host interactions and immune reaction modulation. Vet Dermatol. 2007;18(4):197-204.
24. Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol. 2009;9(7):465-79.
25. Napoletano C, Zizzari IG, Rughetti A, Rahimi H, Irimura T, Clausen H, et al. Targeting of macrophage galactose-type C-type lectin (MGL) induces DC signaling and activation. Eur J Immunol. 2012;42(4):936-45.
26. Kingeter LM, Lin X. C-type lectin receptor-induced NF-kappaB activation in innate immune and inflammatory responses. Cell Mol Immunol. 2012;9(2):105-12.
27. van Vliet SJ, van Liempt E, Saeland E, Aarnoudse CA, Appelmelk B, Irimura T, et al. Carbohydrate profiling reveals a distinctive role for the C-type lectin MGL in the recognition of helminth parasites and tumor antigens by dendritic cells. Int Immunol. 2005;17(5):661-9.
28. Yuita H, Tsuiji M, Tajika Y, Matsumoto Y, Hirano K, Suzuki N, et al. Retardation of removal of radiation-induced apoptotic cells in developing neural tubes in macrophage galactose-type C-type lectin-1-deficient mouse embryos. Glycobiology. 2005;15(12):1368-75.
29. van Vliet SJ, Aarnoudse CA, Broks-van den Berg VC, Boks M, Geijtenbeek TB, van Kooyk Y. MGL-mediated internalization and antigen presentation by dendritic cells: a role for tyrosine-5. Eur J Immunol. 2007;37(8):2075-81.
30. Tsuiji M, Fujimori M, Ohashi Y, Higashi N, Onami TM, Hedrick SM, et al. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. J Biol Chem. 2002;277(32):28892-901.
31. Higashi N, Fujioka K, Denda-Nagai K, Hashimoto S, Nagai S, Sato T, et al. The macrophage C-type lectin specific for galactose/N-acetylgalactosamine is an endocytic receptor expressed on monocyte-derived immature dendritic cells. J Biol Chem. 2002;277(23):20686-93.
32. Singh SK, Streng-Ouwehand I, Litjens M, Weelij DR, Garcia-Vallejo JJ, van Vliet SJ, et al. Characterization of murine MGL1 and MGL2 C-type lectins: distinct glycan specificities and tumor binding properties. Mol Immunol. 2009;46(6):1240-9.
33. Lopez-Olmos V, Perez-Nasser N, Pinero D, Ortega E, Hernandez R, Espinoza B. Biological characterization and genetic diversity of Mexican isolates of Trypanosoma cruzi. Acta Trop. 1998;69(3):239-54.
34. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem. 1978;87(1):206-10.
35. Onami TM, Lin MY, Page DM, Reynolds SA, Katayama CD, Marth JD, et al. Generation of mice deficient for macrophage galactose- and N-acetylgalactosamine-specific lectin: limited role in lymphoid and erythroid homeostasis and evidence for multiple lectins. Mol Cell Biol. 2002;22(14):5173-81.
36. Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. Simplified mammalian DNA isolation procedure. Nucleic Acids Res. 1991;19(15):4293.
37. Gupta S, Garg NJ. TcVac3 induced control of Trypanosoma cruzi infection and chronic myocarditis in mice. PLoS One. 2013;8(3):e59434.
38. Ropert C, Ferreira LR, Campos MA, Procopio DO, Travassos LR, Ferguson MA, et al. Macrophage signaling by glycosylphosphatidylinositol-anchored mucin-like glycoproteins derived from Trypanosoma cruzi trypomastigotes. Microbes Infect. 2002;4(9):1015-25.
39. Sastry MV, Banarjee P, Patanjali SR, Swamy MJ, Swarnalatha GV, Surolia A. Analysis of saccharide binding to Artocarpus integrifolia lectin reveals specific recognition of T-antigen (beta-D-Gal(1----3)D-GalNAc). J Biol Chem. 1986;261(25):11726-33.
40. Kumar S, Tarleton RL. Antigen-specific Th1 but not Th2 cells provide protection from lethal Trypanosoma cruzi infection in mice. J Immunol. 2001;166(7):4596-603.
41. Mylonas KJ, Nair MG, Prieto-Lafuente L, Paape D, Allen JE. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. J Immunol. 2009;182(5):3084-94.
42. Espinoza-Jimenez A, Peon AN, Terrazas LI. Alternatively activated macrophages in types 1 and 2 diabetes. Mediators Inflamm. 2012;2012:815953.
43. Tomioka H, Tatano Y, Maw WW, Sano C, Kanehiro Y, Shimizu T. Characteristics of suppressor macrophages induced by mycobacterial and protozoal infections in relation to alternatively activated M2 macrophages. Clin Dev Immunol. 2012;2012:635451.
44. Arnold CE, Whyte CS, Gordon P, Barker RN, Rees AJ, Wilson HM. A critical role for SOCS3 in promoting M1 macrophage activation and function in vitro and in vivo. Immunology. 2013.
45. Van Dyken SJ, Locksley RM. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. Annu Rev Immunol. 2013;31:317-43.
46. El Fiky A, Perreault R, McGinnis GJ, Rabin RL. Attenuated expression of interferon-beta and interferon-lambda1 by human alternatively activated macrophages. Hum Immunol. 2013.
47. Terrazas CA, Alcantara-Hernandez M, Bonifaz L, Terrazas LI, Satoskar AR. Helminth-excreted/secreted products are recognized by multiple receptors on DCs to block the TLR response and bias Th2 polarization in a cRAF dependent pathway. FASEB J. 2013;27(11):4547-60.
48. Raes G, Brys L, Dahal BK, Brandt J, Grooten J, Brombacher F, et al. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. J Leukoc Biol. 2005;77(3):321-7.
49. Sancho D, Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. Annu Rev Immunol. 2012;30:491-529.
50. Lang R, Schoenen H, Desel C. Targeting Syk-Card9-activating C-type lectin receptors by vaccine adjuvants: findings, implications and open questions. Immunobiology. 2011;216(11):1184-91.
51. van Vliet SJ, Bay S, Vuist IM, Kalay H, Garcia-Vallejo JJ, Leclerc C, et al. MGL signaling augments TLR2-mediated responses for enhanced IL-10 and TNF-alpha secretion. J Leukoc Biol. 2013;94(2):315-23.
52. Napoletano C, Rughetti A, Agervig Tarp MP, Coleman J, Bennett EP, Picco G, et al. Tumor-associated Tn-MUC1 glycoform is internalized through the macrophage galactose-type C-type lectin and delivered to the HLA class I and II compartments in dendritic cells. Cancer Res. 2007;67(17):8358-67.
53. van Liempt E, van Vliet SJ, Engering A, Garcia Vallejo JJ, Bank CM, Sanchez-Hernandez M, et al. Schistosoma mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. Mol Immunol. 2007;44(10):2605-15.
54. van Vliet SJ, Saeland E, van Kooyk Y. Sweet preferences of MGL: carbohydrate specificity and function. Trends Immunol. 2008;29(2):83-90.
55. Stafford JL, Neumann NF, Belosevic M. Macrophage-mediated innate host defense against protozoan parasites. Crit Rev Microbiol. 2002;28(3):187-248.
56. Brener Z, Gazzinelli RT. Immunological control of Trypanosoma cruzi infection and pathogenesis of Chagas' disease. Int Arch Allergy Immunol. 1997;114(2):103-10.
57. Reyes JL, Terrazas LI, Espinoza B, Cruz-Robles D, Soto V, Rivera-Montoya I, et al. Macrophage migration inhibitory factor contributes to host defense against acute Trypanosoma cruzi infection. Infect Immun. 2006;74(6):3170-9.
58. Li D, Romain G, Flamar AL, Duluc D, Dullaers M, Li XH, et al. Targeting self- and foreign antigens to dendritic cells via DC-ASGPR generates IL-10-producing suppressive CD4+ T cells. J Exp Med. 2012;209(1):109-21.

Articulo 2: Tonathiu Rodriguez, Thalia Pacheco-Fernández, Alicia Vázquez-Mendoza, Oscar Nieto-Yañez, Imelda Juárez-Avelar, José L. Reyes, Luis I. Terrazas and Miriam Rodriguez-Sosa 1. 2020. MGL1 Receptor Plays a Key Role in the Control of T. cruzi Infection by Increasing Macrophage Activation through Modulation of ERK1/2, c-Jun, NF-kB and NLRP3 Pathways. Cells. IF 5.6.

cells

MDPI

Article

MGL1 Receptor Plays a Key Role in the Control of *T. cruzi* Infection by Increasing Macrophage Activation through Modulation of ERK1/2, c-Jun, NF-κB and NLRP3 Pathways

Tonathiu Rodriguez [1], Thalia Pacheco-Fernández [1], Alicia Vázquez-Mendoza [2], Oscar Nieto-Yañez [1], Imelda Juárez-Avelar [1], José L. Reyes [1], Luis I. Terrazas [1,3] and Miriam Rodriguez-Sosa [1,*]

1 Unidad de Biomedicina (UBIMED), Facultad de Estudios Superiores Iztacala (FES-Iztacala), Universidad Nacional Autónoma de México (UNAM). Tlalnepantla, Estado de México 54090, Mexico; tonathiurh@hotmail.com (T.R.); silaht16@gmail.com (T.P.-F.); biol.oscarnieto@gmail.com (O.N.-Y.); imelda_juarez@yahoo.com (I.J.-A.); jlreyes@iztacala.unam.mx (J.L.R.); literrazas@unam.mx (L.I.T.)

2 Carrera de Optometría, FES-Iztacala, UNAM. Tlalnepantla, Estado de México 54090, Mexico; aliciavm@ired.unam.mx

3 Laboratorio Nacional en Salud, FES-Iztacala, UNAM. Tlalnepantla, Estado de México 54090, Mexico

* Correspondence: rodriguezm@unam.mx; Tel.: +52-55-5623-1333 (ext. 39789)

Received: 13 November 2019; Accepted: 19 December 2019; Published: 1 January 2020

Abstract: Macrophage galactose-C type lectin (MGL)1 receptor is involved in the recognition of *Trypanosoma cruzi* (*T. cruzi*) parasites and is important for the modulation of the innate and adaptive immune responses. However, the mechanism by which MGL1 promotes resistance to *T. cruzi* remains unclear. Here, we show that MGL1 knockout macrophages (MGL1$^{-/-}$ Mϕ) infected in vitro with *T. cruzi* were heavily parasitized and showed decreased levels of reactive oxygen species (ROS), nitric oxide (NO), IL-12 and TNF-α compared to wild-type macrophages (WT Mϕ). MGL1$^{-/-}$ Mϕ stimulated in vitro with *T. cruzi* antigen (*Tc*Ag) showed low expression of TLR-2, TLR-4 and MHC-II, which resulted in deficient splenic cell activation compared with similar co-cultured WT Mϕ. Importantly, the activation of p-ERK1/2, p-c-Jun and p-NF-κB p65 were significantly reduced in MGL1$^{-/-}$ Mϕ exposed to *Tc*Ag. Similarly, procaspase 1, caspase 1 and NLRP3 inflammasome also displayed a reduced expression that was associated with low IL-β production. Our data reveal a previously unappreciated role for MGL1 in Mϕ activation through the modulation of ERK1/2, c-Jun, NF-κB and NLRP3 signaling pathways, and to the development of protective innate immunity against experimental *T. cruzi* infection.

Keywords: C-type lectin-like receptors; macrophage galactose-C type lectin; mouse MGL; *Trypanosoma cruzi*; PRRs; innate immunity response

1. Introduction

The C-type lectins are a superfamily of more than 1000 proteins that are identified by having one or more characteristic C-type lectin-like domains (CTLDs) [1,2]. These molecules were originally named for their ability to bind carbohydrates in a calcium (Ca^{+})-dependent manner through conserved residues within the CTLDs. However, the CTLDs of many C-type lectins lack the components required for Ca^{2+}-dependent carbohydrate recognition and can recognize a broader repertoire of ligands, including proteins, lipids and inorganic molecules [3].

In mammals, C-type lectins are found as secreted molecules or as transmembrane proteins known as transmembrane C-type lectin receptors (CLRs). Traditionally, it is widely accepted that CLRs that are

expressed in antigen presenting cells (APCs) play an important role in the recognition, internalization of self (endogenous) and non-self (exogenous) ligands and regulate the routing of the internalized antigens to MHC-I or MHC-II-loading compartments for their further presentation to T cells, thereby creating a specific immune response [4,5]. However, it has recently been shown that CLRs also play an important role in promoting diverse physiological functions, such as the modulation of cellular, developmental, homeostatic and immunological responses [6,7].

Protozoa parasites possess numerous glycosylated structures that are capable of activating the innate immune response by binding to pattern recognition receptors (PRRs), such as toll like receptors (TLRs) and CLRs such as the mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), MGL and Dectin-1, among others [2,8]. Previous reports have shown that CLRs cooperate with TLRs to activate intracellular signaling pathways upon sensing pathogen-derived antigens [9–11]. For example, Dectin-1 and MR expressed in Mϕ play a crucial role in the microbicidal response by inducing reactive oxygen species (ROS) production against the protozoan *Leishmania infantum* [12]. Despite their importance, many of the CLRs involved in cellular activities are poorly characterized, and their contribution and underlying mechanism remain incompletely understood.

Macrophage galactose-C type lectin is a CLR that is selectively expressed as a homo-oligomer on APCs such as immature dendritic cells (DC) and Mϕ in humans and mice [11]. The carbohydrate recognition domain of MGL binds with high affinity to glyproteins expressing terminal galactose (Gal) and N-acetylgalactosamine (GalNAc) residues [13,14]. In mice, there are two homologs of human MGL, MGL1 and MGL2 [15]. MGL1 shares significant sequence homology with human MGL, recognizes terminal Gal and Lewis X structure residues and can mediate glycoprotein endocytosis [16,17], whereas MGL2 recognizes α-and β-GalNAc and does not interact with Lewis X structures [18]. The variation and distribution of MGL1 and MGL2 in healthy mouse cells shows a significant portion of the MGL1 single positive cells in bone marrow (BM), peritoneal and spleen cells. Specifically, a portion of conventional DC (cDC) co-express MGL1 and MGL2, while another portion of cDCs and plasmocytoid DCs (pDC) only express MGL1 (mostly cDC). The peritoneal exudate Mϕ (PE-Mϕ) and BMMϕ expresses significant levels of MGL1, while MGL2 expression is low in PE-Mϕ and almost absent in BMMϕ [19].

The intracellular parasite *Trypanosoma cruzi* is the causative agent of Chagas' disease, an important health problem in Latin America that is becoming an emerging global public health problem as a result of migration and global climate change [20]. Abundant amounts of mucin-like glycoproteins are present on this parasite's surface. These glycoconjugates contain approximately 60% carbohydrates, most of which are anchored to glycosylphosphatidylinositol (GPI)- and glycoinositolphospholipid (GIPL)-mucin molecules [21]. We have previously reported that most *Tc*Ag bind to lectin Jacalin, which recognizes Gal residues; therefore, *Tc*Ag are glycosylated with Gal residues. In addition, we showed that MGL1$^{-/-}$ mice were highly susceptible to *T. cruzi* infection, and they developed higher parasitemia and mortality rates than WT mice [22]. Since *Tc*Ag contains Gal residues and MGL1$^{-/-}$ Mϕ showed an impaired ability to eliminate this parasite, we hypothesized that MGL1 could play an important role in optimal Mϕ activation. The mechanism by which the MGL1 receptor works has not been identified; therefore, we aimed to clarify the mechanism by which the MGL1 activates Mϕ against *T. cruzi* infection.

Here, we show that the absence of MGL1 led to impaired Mϕ activation and we also provide additional evidence to support that MGL1$^{-/-}$ Mϕ had significantly reduced phosphorylation of subunit p65 of nuclear factor (p-NF)-κB, extracellular signal-regulated kinase 1/2 (p-ERK1/2) and transcription factor c-Jun (p-c-Jun), and decreased expression levels of the nucleotide-binding domain leucine-rich repeats family protein (NLRP3) in *T. cruzi* infection.

2. Materials and Methods

2.1. Mice

Six- to eight-week-old male MGL1$^{-/-}$ mice on a C57BL/6 genetic background (donated by Glycomics Consortium, USA) were backcrossed for more than 10 generations [23]. WT C57BL/6 background mice were purchased from Harlan (Invigo, Mexico City, Mexico). Mice were maintained in a pathogen free environment at the FES-Iztacala, UNAM animal facilities. Genotyping of MGL1$^{-/-}$ mice was routinely performed on DNA isolated from tail snips using a polymerase chain reaction (PCR) procedure [24]. The PCR were performed using the following primers: MGL1: forward 5′-CTTG GTCCCAGATCCGTATC-3′ and reverse 5′-ATGTCATGACTCAGGATC-3′; Neomycin (NEO): forward 5′-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3′ and reverse 5′-AAGAACTCGTCA AGAAGGCGATAGAAGGCG-3′ (All synthesized by Sigma-Aldrich, Mexico City, Mexico). PCR for the amplification of MGL and NEO was performed with Taq DNA polymerase (Ampliqon, Bioreagents and Molecular Diagnostics) following the manufacturer's instructions. A PCR fragment of 714 bp, corresponding to MGL, or 492 bp, corresponding to NEO, was visualized to identify WT or MGL1$^{-/-}$ mice, respectively. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel and were viewed under UV light (Bio-Rad, Mexico City, Mexico).

All experimental procedures using animals were designed to minimize suffering and the number of subjects used. These studies were conducted in accordance with the ethical standards approved and carried out under strict accordance with the guidelines for the Care and Use of Laboratory Animals adopted by the U.S. National Institutes of Health, and the Mexican Regulation of Animal Care and maintenance (NOM-062ZOO-1999, 2001). And it was revised and approved by the Ethics Committee at FES-Iztacala, UNAM (CE/FESI/062019/1311).

2.2. Parasites

The Mexican *T. cruzi* TBAR/MX/0000/Queretaro strain belonging to DTU TcI was used in this work. Epimastigotes of *T. cruzi* were cultured at 28 °C in biphasic culture with brain heart infusion broth, agar and dextrose (Sigma-Aldrich, Mexico City, Mexico), and in the liquid phase with saline solution supplemented with 5% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) with 100 U of penicillin/streptomycin (all from GIBCO-BRL, Grand Island, NY, USA).

2.3. Soluble T. cruzi Lysate Antigen (TcAg)

Culture-derived epimastigotes were obtained and washed three times in phosphate buffered saline (PBS) by centrifugation at 1300× *g* for 10 min. The obtained pellet was sonicated six times for 10 s each at 50 W using a sonic Dimem-brator 300 (Thermo Fisher Scientific, Waltham, MA, USA) in the presence of protease inhibitors (Sigma-Aldrich). Parasite lysis was confirmed using a microscope. Parasite lysates were then centrifuged at 20,000× *g* for 30 min at 4 °C, and PBS-soluble antigens were stored at −70 °C until use. The protein concentration was determined using a Bradford protein kit (Sigma-Aldrich).

2.4. Cell preparations and T. cruzi Infection In Vitro

Peritoneal exudate cells (PECs) were obtained from the peritoneal cavity of MGL1$^{-/-}$ and WT mice under sterile conditions using 10 mL of ice-cold Hank´s balanced salt solution (Microlab, Mexico City, Mexico). Following two washes with Hank's balanced solution, red blood cells were lysed by resuspending the cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride, all from Sigma-Aldrich). The viable cells were counted using the trypan blue exclusion method (routinely exceeding 95%) with a Neubauer hemocytometer (Sigma-Aldrich). PECs were adjusted to a concentration of 5×10^6 cells/mL in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U of penicillin/streptomycin, and 2 mM glutamine (all from GIBCO-BRL), and cultured in 24-well plates (Costar, Bedford, MA, USA). After 2 h at 37 °C and 5% CO_2, non-adherent

cells were removed by washing with warm DMEM. Adherent cells (PE-Mϕ) were removed from the plate by washing with 5 mM ethylenediaminetetraacetic acid (EDTA) in warm PBS and then adjusted to a concentration of 1×10^6 cells/mL. PE-Mϕ were allowed to adhere on coverslips in 24-well flat-bottomed culture plates for 2 h and then were infected with culture-derived epimastigotes at a ratio of 10:1 (parasites: Mϕ) in supplemented DMEM. After 2 h of incubation, cells were washed with DMEM to remove the extracellular parasites; coverslips were removed, and stained with Giemsa (Sigma-Aldrich). A minimum of 100 cells in different microscopic fields were examined by a light Zeiss AXIO Vert A1 microscope (Carl Zeiss, Berlin, Germany), and the percentage of infected cells and the number of amastigotes per infected cell were determined in triplicate in a double-blind manner. Five independent experiments were analyzed. Supernatants were collected and stored at −70 °C until use for the quantification of NO and cytokine production (IL-1β, IL-10, IL-12 and TNF-α).

2.5. Reactive Oxygen Species Activity Assay

PE-Mϕ from MGL1$^{-/-}$ and WT mice grown on coverslips as mentioned above were exposed to culture-derived epimastigotes at a ratio of 10:1 (parasites: 1 Mϕ) or *Candida albicans* (*C. albicans*) (5×10^6 cells/mL) for 2 h at 37 °C and 5% CO_2. Next, ROS production was measured by the nitrobluetetrazolium (NBT) test, by adding 100 µL of 0.1% NBT (Sigma-Aldrich) to a final volume of 1 mL per well with DMEM. The resting plate was incubated at 37 °C for 1 h. The coverslips were rinsed with saline to wash away excess NBT. Mϕ were stained with 0.5% safranin for 7 min and washed with distilled water. Finally, the coverslips were mounted on glass slides with resin, and 100 cells in different fields were examined under a light microscope (Zeiss AXIO Vert A1 microscope; Carl Zeiss, Berlin, Germany); the percentage of ROS positive cells was determined in a double-blinded fashion.

2.6. Detection of Nitric Oxide and Cytokines Production

PE-Mϕ from MGL1$^{-/-}$ and WT mice were grown in 24-well flat-bottomed plate, as mentioned above, were left untreated or treated with lipopolysaccharide (LPS; 100 ng/mL, *Escherichia coli* 0111:B4, Sigma-Aldrich), IFN-γ (10 U/mL, Peprotech, Mexico City, Mexico), or LPS+IFN-γ (100 ng/mL + 10 U/mL) for 24 h. Then, they were infected with culture-derived epimastigotes at a ratio of 10:1 (parasites: Mϕ) for 2 h at 37 °C and 5% CO_2. Cells were washed with DMEM to remove the extracellular parasites, and the plates were incubated for an additional 24 h. Supernatant from MGL1$^{-/-}$ PE-Mϕ and WT PE-Mϕ cultures, were assayed for nitric oxide (NO) production by the Griess reaction adapted for 96-well plates (Costar) [25]. Briefly, 50 µL of culture supernatant was mixed with an equal volume of Griess reagent and incubated for 10 min at room temperature in the dark. The concentration was determined by extrapolating the optical density from each sample to a standard curve of sodium nitrite. The absorbance was measured at 570 nm in Epoch microplate spectrophotometer (BioTEk, Winooski, VT, USA). To determine IL-12, TNF-α, IL-10 and IL-1β levels by using commercially available enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (Peprotech, Mexico City, Mexico). The optical density (OD) was measured using an Epoch microplate spectrophotometer (BioTEk) at 405 nm.

2.7. Viability of Internalized T. cruzi

To test the trypanocidal capacity of both MGL1$^{-/-}$ and WT Mϕ, parasite proliferation was examined as previously described [26]. Briefly, Mϕ from MGL1$^{-/-}$ and WT mice were obtained as described in Section 2.4 and adjusted to 1×10^6 cells/mL. These cells were seeded in 24-well flat-bottom culture plates, and incubated for 2 h at 37 °C and 5% CO_2. Mϕ were washed with DMEM, and divided into groups as follows: non-stimulated or stimulated with LPS (100 ng/mL), IFN-γ (10 U/mL) or LPS+IFN-γ (100 ng/mL + 10 U/mL) for 24 h. Then, Mϕ were infected with culture-derived epimastigotes at a ratio of 10:1 (parasites: Mϕ) in supplemented DMEM. After 2 h, Mϕ were washed twice with DMEM to remove non-internalized parasites, and fresh supplemented DMEM was added to the culture and Mϕ were incubated for another 24h. After this time supernatants were collected

and stored at −70 °C until use for NO quantification and cytokine production (IL-1β, IL-10, IL-12 and TNF-α). Mϕ were lysed using 0.01% sodium dodecyl sulfate (SDS) in 100 mL of warm PBS for 30 min and pipetted up and down 5–10 times. Released amastigotes from the Mϕ were harvested and centrifuged at 1300× *g*. The pellet was resuspended in 600 µL of supplemented DMEM medium, and aliquots (150 µL) of suspension were seeded into 96-well flat-bottom culture plates (Costar) at 37 °C and 5% CO_2 for 72 h. Eighteen hours prior to culture termination, 0.5 µCi of tritiated thymidine ([^{3}H]TdR, 185 GBb/mmol activity; Amersham, Aylesbury, UK) was added to each well. The cells were harvested on fiberglass paper (PerkinElmer, Billerica, MA, USA), and the counts per minute (CPM) were quantified using a liquid scintillation counter Trilux 1450 Microbeta (Tomtec, Hamden, CT, USA). The Mϕ trypanocidal activity was measured as a reduction in the incorporation of [^{3}H]TdR by surviving amastigotes recovered from the Mϕ that become into epimastigotes in the cell-free medium.

2.8. Flow Cytometry Analysis

PE-Mϕ from MGL1$^{-/-}$ and WT mice were obtained and stimulated with *Tc*Ag (25 µg/mL) for 24 or 48 h. These cells were incubated with anti-mouse FcγR antibody (CD16/CD32) in staining buffer (1× PBS, 2% FBS, 1% NaN3) for 15 m, followed by incubation for 30 m at 4 °C with FITC-conjugated anti-F4/80, PE-conjugated anti-MGL1 and APC-conjugated anti-MGL2 antibodies (BioLegend, SD, CA). For quantification of costimulatory molecule expression, MGL1$^{-/-}$ and WT PE-Mϕ and BMMϕ were stimulated in vitro with LPS (100 ng/mL) or *Tc*Ag (25 µg/mL) for 24 h or infected with culture-derived epimastigotes for 2 h (ratio 1:10). The cells were incubated with the following fluorochrome-conjugated Abs: Pacific blue anti-F4/80, PerCP/Cy5.5 anti-CD11b, PE anti-TLR-4, PE anti-MHC-II, FITC anti-TLR-2, FITC anti-CD40 and FITC anti-CD80 (all from BioLegend), as well as the negative control. Mϕ were washed three times with FACS buffer and fixed in 0.8% paraformaldehyde before acquisition and analysis (Attune NxT, Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Co-culture of Mϕ and Splenic Cells

To examine the antigen presenting capacity of MGL1$^{-/-}$ Mϕ, Mϕ were co-cultured with splenocytes as follows: PE-Mϕ from MGL1$^{-/-}$ or WT mice were obtained and adjusted to 1×10^6 cells/mL as described above. PE-Mϕ were seeded (100 µL) in 96-well flat-bottom culture plates (Costar) and stimulated with 100 µL of *Tc*Ag (25 µg/mL) at 37 °C and 5% CO_2. Two hours later, PE-Mϕ were washed three times to remove the non-phagocytosed antigen. Splenocytes from WT mice infected with *T. cruzi* for 21 days were added at a ratio of 1:10 (Mϕ: splenocytes; 100 µL of splenocytes adjusted to a concentration of 10×10^6 cells/mL). Co-cultures were maintained at 37 °C and 5% CO_2 for 72 h, and then 0.5 µCi/well of [^{3}H]-thymidine (185 GBb/mmol activity; Amersham, Aylesbury, UK) was added and incubated for additional 18 h. The plate was harvested on fiberglass paper (PerkinElmer) using a 96-well harvester (Tomtec, Toku, Finland), and CPM were quantified using a liquid scintillation Trilux 1450 Microbeta counter (Tomtec).

2.10. Protein Levels of NF-κB, P38, ERK1/2 and NLRP3 Signaling Pathways Detected by Western Blotting

Murine BMMϕ were generated using tibias and femurs aseptically removed from MGL1$^{-/-}$ and WT mice as previously described [27]. Briefly, bone ends were cut and flushed with 10 mL of sterile PBS. The obtained cell suspension was centrifuged at 1300× *g* for 10 min at 4 °C. Cells were adjusted to a concentration of 4×10^6 cells/mL in Mϕ differentiating medium containing supplemented DMEM (20% FBS) and 50 ng/mL murine macrophage colony-stimulating factor (M-CSF) (Biotech, BG, DE). Two milliliters of cell suspension was seeded into each well of a 6-well plate and incubated at 37 °C in 5% CO_2. After 72 h, 1 mL of differentiating medium was added to each well. Cells were allowed to differentiate for 7 days. BMMϕ were washed twice, adjusted to a concentration of 4×10^6 cells/mL and stimulated with LPS (100 ng/mL) or *Tc*Ag (25 µg/mL) for 0, 5, 15 and 30 min. BMMϕ protein was extracted using Laemmli buffer (containing, 92 mM Tris (pH 6.8), 18% glycerol, 1.8% SDS, 0.02% bromophenol blue and 2% β-mercaptoethanol (all from Sigma-Aldrich) with protease

and phosphatase inhibitors (Roche Diagnostic, Basel Switzerland) according to the manufacturer's instructions. The samples were centrifuged at 700× *g* for 5 min at 4 °C, and the protein concentration was determined using a Bradford assay (Sigma-Aldrich). Protein samples (15 µg) were separated by 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis at 80 V and were transferred to immobilon-P membranes (0.22 µM, Millipore, Bedford, MA, USA) by electroblotting. The membranes were blocked for 2 h at room temperature in Tris-buffered saline-Tween 20 (TBST) supplemented with 5% *w/v* bovine serum albumin (Sigma-Aldrich). Subsequently, the membrane was washed three times with TBST and incubated at 4 °C overnight on a shaker plate with the following primary antibodies: GAPDH (as housekeeping protein), NF-κB p65, p-NF-κB p65, p38 MAPK, p-p38 MAPK, p44/42 MAPK (ERK1/2), p-p44/42 MAPK (p-ERK1/2) and NLRP3 following the manufacturer's protocol (Cell Signaling, Danvers, MA, USA). After washing the membrane with TBST four times, an alkaline phosphatase-conjugated secondary antibody in TBST was added (dilution 1:5000; Cell Signaling) and incubated for 2 h at room temperature. The membrane was washed with TBST four times, the signal was revealed using Super Signal West Femto (Thermo Fisher Scientific) and then scanned and analyzed using a fluorescent Odyssey infrared scanner (LI-COR, Lincoln, NE, USA).

2.11. Statistical Analysis

Comparisons between the WT and MGL1$^{-/-}$ groups were made using the unpaired Student's t-test or one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism Program version 6.0 (GraphPad Software, La Jolla, CA, USA). All data are shown as the mean ± standard error of the mean (SEM) for at least two or three independent experiments. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Trypanosoma cruzi Antigens Induce High Expression of MGL1 and Moderate Expression of MGL2 in PE-Mϕ

To verify the expression of MGL1 and MGL2 receptors, PE-Mϕ (F4/80^{+}) from WT and MGL1$^{-/-}$ mice were analyzed by flow cytometry after stimulation in vitro for 24 and 48 h with *Tc*Ag. As Denda-Nagai et al. reported [19], WT PE-Mϕ showed surface expression of the MGL1 and no expression of MGL2 receptors at baseline (Figure 1a,b; WT-Mϕ, non-stimulated, gray shadow). After stimulation with *Tc*Ag for 24 or 48 h, MGL1 expression increased significantly while MGL2 expression increased moderately (Figure 1a,b; WT-Mϕ, gray shadow). These observations indicate that MGL1 receptor is highly expressed, and it is mostly induced by the *Tc*Ag in WT PE-Mϕ, compared to MGL2 expression.

PE-Mϕ from MGL1$^{-/-}$ mice did not show expression of MGL1, but moderate levels of MGL2 expression were observed at baseline (Figure 1a,b; MGL1$^{-/-}$ Mϕ, non-stimulated, solid line). Importantly, after *Tc*Ag stimulation, MGL1$^{-/-}$ PE-Mϕ did not show expression of MGL1, whereas MGL2 was slightly elevated (Figure 1a,b; MGL1$^{-/-}$ Mϕ, solid line). These results demonstrate that PE-Mϕ from MGL1$^{-/-}$ mice are unable to upregulate significantly MGL2 in response to *Tc*Ag.

3.2. The MGL1 Receptor Plays a Role in Controlling T. cruzi PE-Mϕ Infection

Pathogens can be recognized by CLRs, and some receptors play a major role in pathogen internalization. We previously demonstrated that MGL1 binds *Tc*Ag, and MGL1$^{-/-}$ mice were found to be more susceptible to in vivo *T. cruzi* infection [2]. Herein, we explored whether the MGL1 receptor plays a role in vitro internalization of *T. cruzi* parasites by PE-Mϕ. To address this question, PE-Mϕ from MGL1$^{-/-}$ and WT mice were infected with culture-derived epimastigotes of *T. cruzi.* The percentage of infected Mϕ was determined 2 h after co-incubation. We observed that MGL1$^{-/-}$ Mϕ exhibited a higher percentage of infected Mϕ (86%) than WT Mϕ (48%) (Figure 2a, $p < 0.05$). Importantly, MGL1$^{-/-}$ Mϕ also exhibited a higher number of internalized parasites per cell. Specifically, 33% of MGL1$^{-/-}$

Mϕ showed 2–3 amastigotes per Mϕ, while 14% of WT Mϕ showed 2–3 amastigotes per Mϕ (Table 1, $p < 0.05$; and Figure 2b).

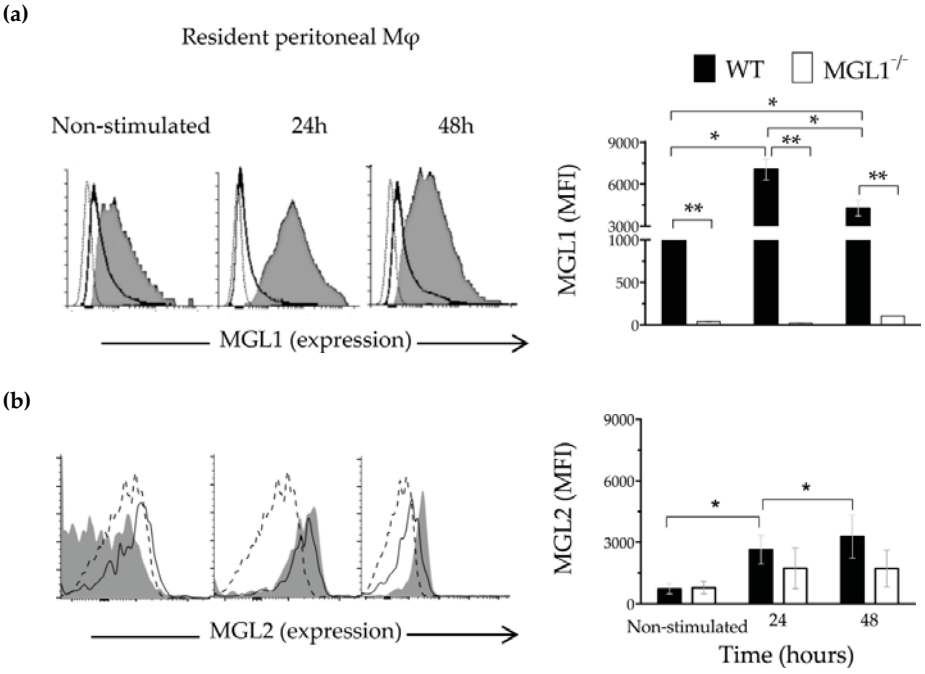


Figure 1. Expression of MGL1 and MGL2 in Mϕ in response to *T. cruzi* antigen. PE-Mϕ (F4/80+) from WT or MGL1$^{-/-}$ mice were stimulated for 24 or 48 h with *Tc*Ag (25 µg/mL). Representative histogram and bar chart of the percentage of PE-Mϕ expressing MGL1 and MGL2 are shown in (**a**) and (**b**), respectively. Dotted line, isotype; gray area, PE-Mϕ from WT mice; solid line, PE-Mϕ from MGL1$^{-/-}$ mice; n = 6 mice per group; * $p < 0.05$ and ** $p < 0.002$.

Table 1. The numbers of Mϕ that exhibit at least one amastigote, and the number of intracellular amastigotes per Mϕ.

		Number of Amastigotes Per Macrophage				
Mϕ Infected/100 cells		**1**	**2–3**	**4**	**5**	**≥6**
WT	48%	29%	14%	4%	1%	0%
MGL1$^{-/-}$	86%*	22%*	33%*	21%*	7%*	3%

Values of * $p < 0.05$ were considered statistically significant compared to WT Mϕ. These are representative of three independent experiments.

As LPS/IFN-γ-treatment induces classically activated Mϕ, which are involved in the control of *T. cruzi* infection, we asked whether pre-activated MGL1$^{-/-}$ Mϕ could control *T. cruzi* infection. To address this question, culture-derived epimastigotes of *T. cruzi* were used to infect untreated MGL1$^{-/-}$ and WT Mϕ or treated with IFN-γ, LPS and LPS/IFN-γ for 24 h. Parasite survival after 24 h was determined by measuring [^{3}H]thymidine incorporation as a marker of parasite proliferation. Untreated WT PE-Mϕ displayed greater [^{3}H]thymidine uptake, indicating parasite proliferation (Figure 2c). As expected, treatment with IFN-γ, LPS or both, IFN-γ and LPS resulted in a significant reduction in parasite survival, as reduced [^{3}H]thymidine uptake was observed in this WT PE-Mϕ (Figure 2c). Interestingly, untreated MGL1$^{-/-}$ PE-Mϕ harbored more surviving *T. cruzi* parasites than untreated WT PE-Mϕ, as shown by the significantly increased uptake of [^{3}H]thymidine ($p < 0.05$%).

Despite being treated with IFN-γ, LPS or both, MGL1$^{-/-}$ PE-Mϕ showed higher levels of parasite proliferation compared with WT PE-Mϕ. MGL1$^{-/-}$ PE-Mϕ therefore displayed an impaired ability to eliminate these parasites, as they maintained a higher [^{3}H]thymidine uptake than their WT PE-Mϕ counterparts. These results suggest that MGL1$^{-/-}$ PE-Mϕ have impaired trypanocidal ability compared to WT PE-Mϕ.

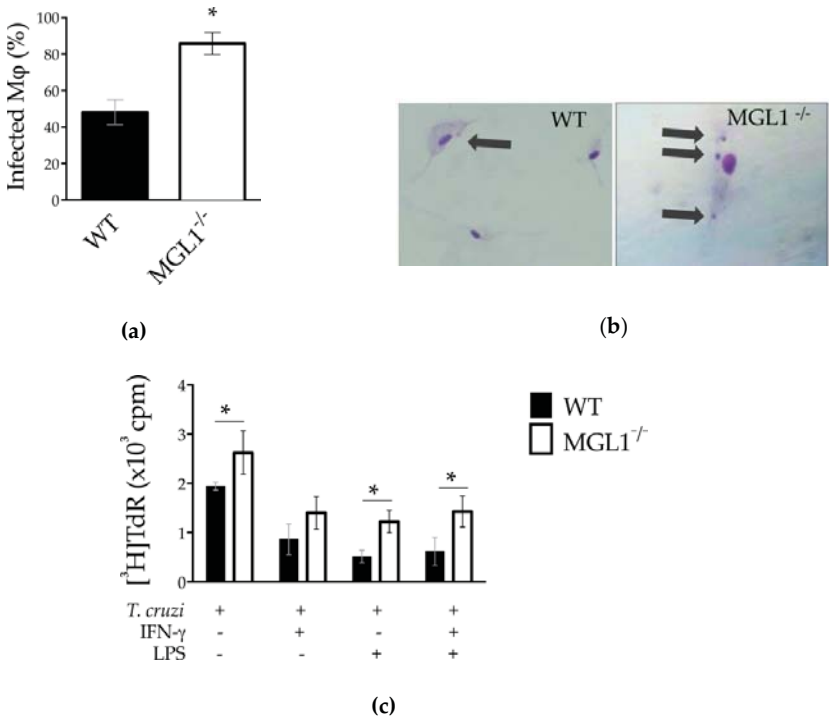


Figure 2. MGL1$^{-/-}$ PE-Mϕ take up more *T. cruzi* parasites. (**a**) Percentage of PE-Mϕ with internal parasites in two hours after infection. (**b**) A representative image of parasites internalized in PE-Mϕ; WT and MGL1$^{-/-}$ PE-Mϕ infected with *T. cruzi* (arrows point to parasites), magnification 40×. (**c**) Mϕ from MGL1$^{-/-}$ and WT mice not treated or treated overnight with IFN-γ, LPS or IFN-γ/LPS followed by exposure to *T. cruzi* at a 10:1 parasite/Mϕ ratio. Parasites that were not taken up were removed at 2 h post-infection, and parasite infection was determined by lysis of the Mϕ and the measurement of parasite proliferation by [^{3}H]thymidine incorporation. The results are shown as the means of replicate samples (± SEM) and are representative of three experiments; * $p < 0.05$.

3.3. MGL1$^{-/-}$ PE-Mϕ Have a Deficient Oxidative Burst, as well as Nitric Oxide and Proinflammatory Cytokine Production during T. cruzi Infection

Reactive oxygen species (ROS) are reactive molecules that include oxygen ions, free radicals and peroxides. ROS are produced as a result of the enzymatic activity that is acquired by the phagosome during its formation. Indeed, the superoxide burst in Mϕ against intracellular parasites represents one of the main antimicrobial mechanisms involved in host defense [28]. To determine whether the MGL1 receptor was associated with ROS production, we performed the NTB test in MGL1$^{-/-}$ and WT Mϕ infected for 2 h with culture-derived epimastigotes of *T. cruzi* or *C. albicans* as a positive control.

We observed a similar increase in ROS production in both MGL1$^{-/-}$ and WT PE-Mϕ infected with *C. albicans*. However, when PE-Mϕ from WT mice were infected with *T. cruzi*, they displayed a significant increase in ROS production, whereas *T. cruzi*-infected Mϕ from MGL1$^{-/-}$ mice did not (Figure 3a).

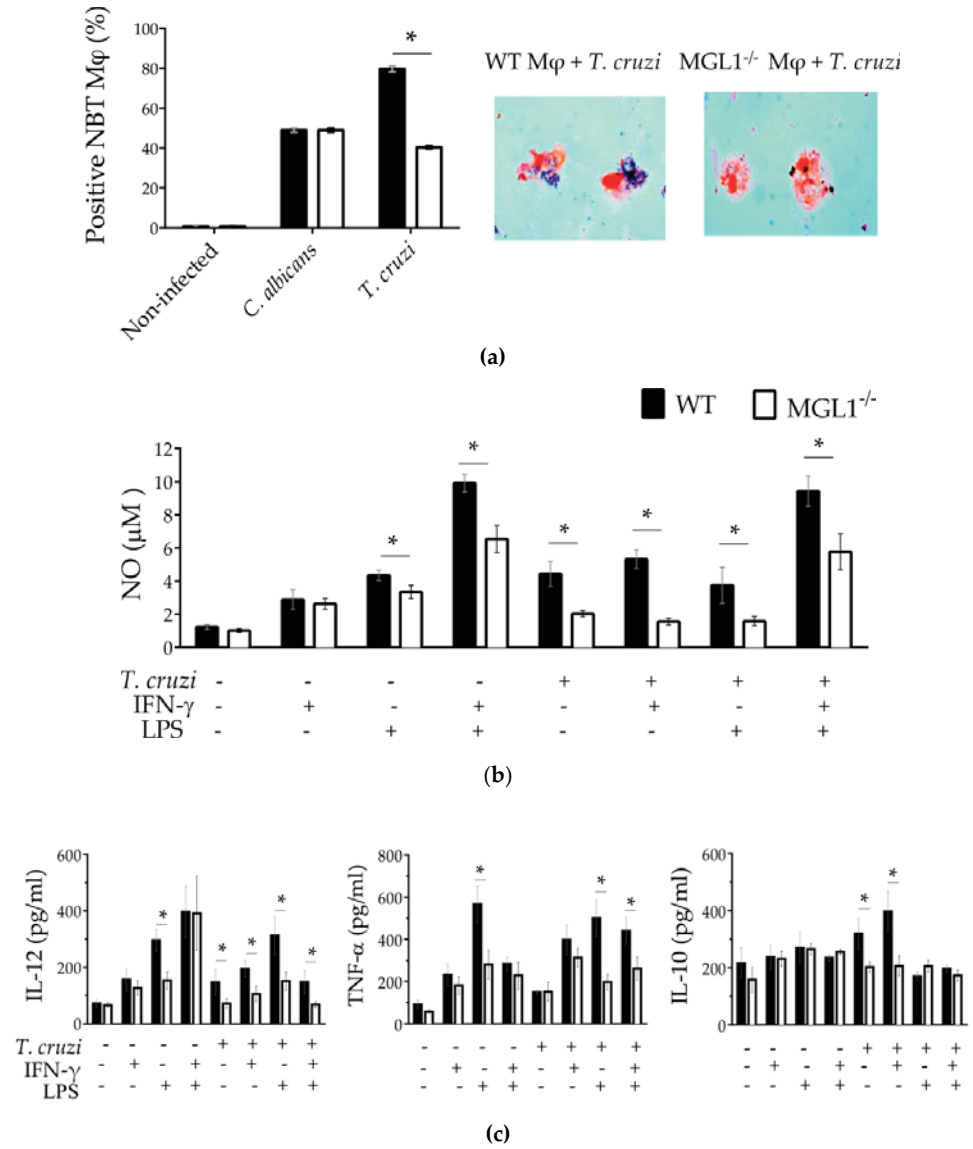


Figure 3. MGL1$^{-/-}$ PE-Mϕ have a deficient ROS, as well as NO and proinflammatory cytokine production during *T. cruzi* infection. (**a**) WT and MGL1$^{-/-}$ PE-Mϕ were infected for 2 h with culture-derived epimastigotes of *T. cruzi* or *Candida albicans* (as positive control), and ROS production was analyzed. (**b**) PE-Mϕ from MGL1$^{-/-}$ and WT mice were not treated or treated for 24 h with IFN-γ, LPS or IFN-γ/LPS followed by infection with epimastigotes of *T. cruzi* at a 10:1 parasite/Mϕ ratio. Non-internalized parasites were removed at 2 h post-infection; supernatants were taken for NO and (**c**) cytokine IL-12, TNF-α and IL-10 quantification. The results are representative of three experiments; * $p < 0.05$.

In addition to ROS production, Mϕ also produce reactive nitrogen species, specially nitric oxide (NO), that, together with IL-12 and TNF-α, plays an important role in controlling the initial infection by *T. cruzi* [21,29]; in contrast, the anti-inflammatory cytokine IL-10 is associated with susceptibility in *T. cruzi* infection [30]. Thus, we tested NO and cytokine production in MGL1$^{-/-}$ and WT PE-Mϕ treated

with LPS, IFN-γ or LPS/IFN-γ for 24 h. These primed PE-Mϕ were later infected with epimastigotes of *T. cruzi* for 2 h, they were washed to remove the extracellular parasites and incubated for an additional 24 h. Supernatants were collected for NO and cytokine quantification.

As expected, WT PE-Mϕ produced slightly elevated NO after IFN-γ stimulation, moderate levels of NO in response to LPS and high levels of NO production in response to LPS/IFN-γ with or without *T. cruzi* infection. However, the MGL1$^{-/-}$ PE-Mϕ did not produce NO in response to *T. cruzi* infection. Indeed, we observed that MGL1$^{-/-}$ PE-Mϕ stimulated with LPS, or LPS/IFN-γ displayed a deficient production of NO with or without *T. cruzi* infection compared with WT PE-Mϕ (Figure 3b).

The proinflammatory cytokines such as IL-12 and TNF-α were induced in response to IFN-γ, LPS or LPS/IFN-γ in WT PE-Mϕ without or with *T. cruzi* infection. Non infected PE-Mϕ from MGL1$^{-/-}$ mice showed similar levels of IL-12 and TNF-α in response to IFN-γ stimulus. Interestingly, the same MGL1$^{-/-}$ PE-Mϕ displayed decreased production of IL-12 and TNF-α in response to LPS. Upon *T. cruzi* infection, MGL1$^{-/-}$ PE-Mϕ stimulated with IFN-γ showed a decreased production of IL-12, and similar levels of TNF-α compared with WT PE-Mϕ (Figure 3c).

IL-10 production showed similar levels in non-infected WT and MGL1$^{-/-}$ PE-Mϕ supernatants, after treatment with IFN-γ, LPS or LPS/IFN-γ. The production of IL-10 increased slightly in WT PE-Mϕ only with infection or with infection + IFN-γ. However, no induction of IL-10 was detected in the MGL$^{-/-}$ PE-Mϕ in *T. cruzi* infection, or with any other stimulus (Figure 3c). These results together support the hypothesis that MGL1 favors the production of ROS, NO and inflammatory cytokines during *T. cruzi* infection.

3.4. MGL1$^{-/-}$ PE-Mϕ Exhibit Deficient Activation of T. cruzi Antigen-Specific Lymphocytes

Next, we analyzed the expression of TLR-2, TLR-4, MHC-II, CD40 and CD80 in MGL1$^{-/-}$ and WT PE-Mϕ stimulated in vitro with LPS or *Tc*Ag or infected with epimastigotes of *T. cruzi* parasites (Figure 4).

Consistent with previous reports that demonstrated that MGL can modulate TLR activation [22,31], we observed significantly lower expression levels of TLR-2 and TLR-4 in MGL1$^{-/-}$ PE-Mϕ compared to WT PE-Mϕ treated with *Tc*Ag or *T. cruzi* parasites (Figure 4a,b). Importantly, we also found reduced expression of MHC-II and CD40 in MGL1$^{-/-}$ Mϕ (Figure 4c,d). However, no significant differences were observed in other costimulatory molecules such as CD80, between MGL1$^{-/-}$ and WT Mϕ treated with *Tc*Ag or *T. cruzi* parasites (Figure 4e). We hypothesized that the deficiency of these molecules could impair the performance of Mϕ as antigen-presenting cells and decrease *T. cruzi*-specific lymphocyte activation. Thus, PE-Mϕ from WT or MGL1$^{-/-}$ mice were pre-loaded with *Tc*Ag to activate *T. cruzi*-specific splenocytes (coming from sensitized mice) in co-cultures. Splenocytes co-cultured with MGL1$^{-/-}$ PE-Mϕ displayed significantly lower proliferation than splenocytes co-cultured with WT PE-Mϕ (Figure 5). These results suggest that lack of MGL1 may also modulate the adaptive response against *T. cruzi*.

3.5. Trypanosoma cruzi Antigens Induce High Expression of MGL1 and Low Expression (Almost Absent) of MGL2 in BMMϕ

It was previously reported that BMMϕ express significant levels of MGL1, while MGL2 is absent in these cells. Therefore, to determine whether the recognition of *Tc*Ag by MGL1 could mediate the activation of Mϕ, we decided to use BMMϕ to establish a possible route of activation.

We first, search for the expression of MGL1 and MGL2 in WT BMMϕ non-stimulated or stimulated with *Tc*Ag by 24 or 48 h. WT BMMϕ showed expression of the MGL1 and almost absent expression of MGL2 at baseline (Figure 6a,b; WT-Mϕ, non-stimulated, gray shadow). After stimulation with *Tc*Ag, MGL1 expression increased significantly, while MGL2 expression did not (Figure 6a,b; WT-Mϕ, gray shadow). These observations suggest that *Tc*Ag may be recognized by MGL1 and favors its up-regulation, whereas MGL2 seems does not participate in this interaction.

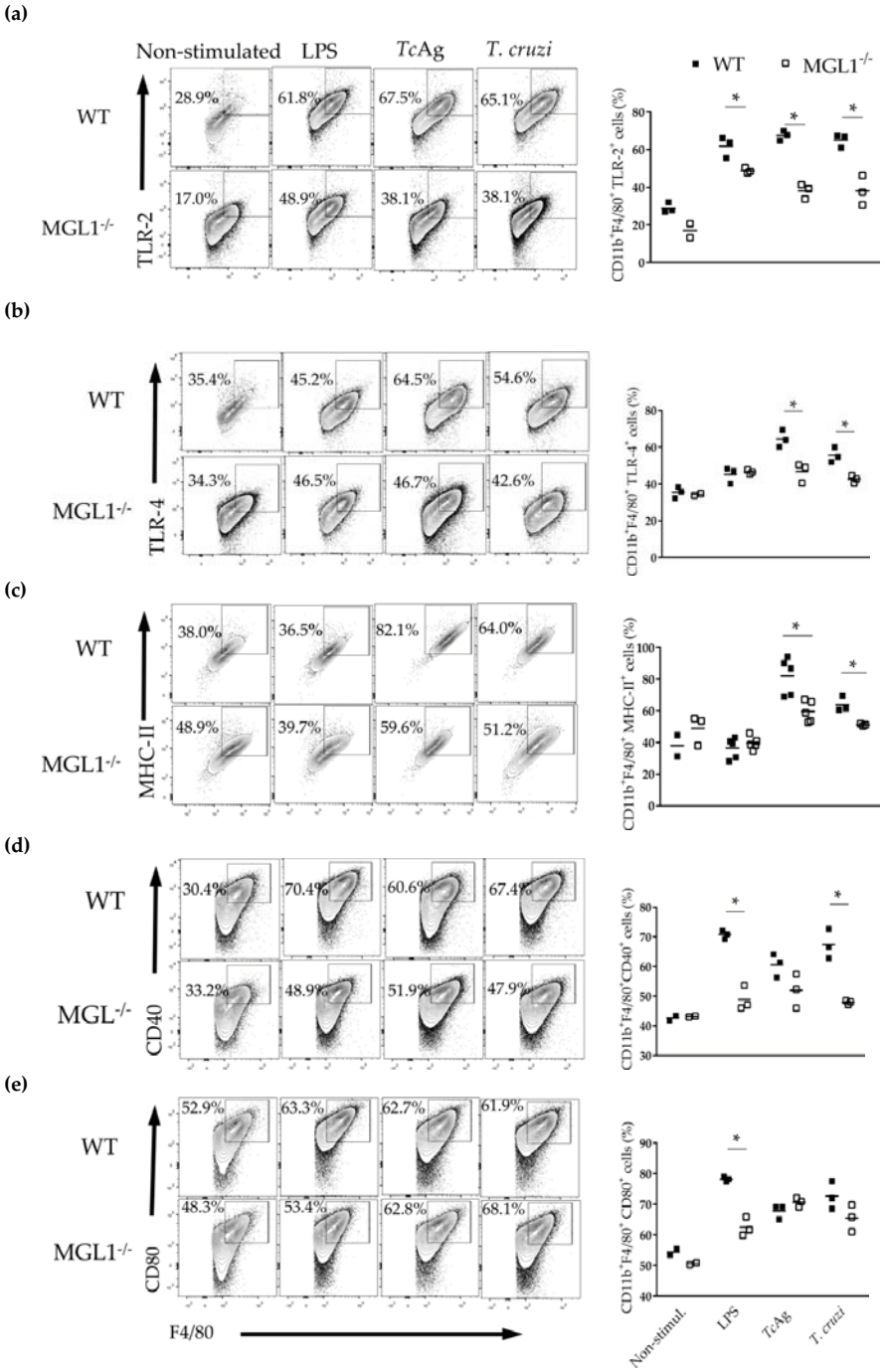


Figure 4. The absence of MGL1 results in reduced expression of TLR2, TLR4, MHCII and CD40 in PE-Mϕ. (**a**–**e**) PE-Mϕ from WT and MGL1$^{-/-}$ mice were stimulated in vitro with LPS (100 ng/mL) or *Tc*Ag (25 μg/mL) or infected with epimastigotes of *T. cruzi* (ratio 1:10) for 24 h. The cells were stained with anti-F4/80, anti-TLR-2, anti-TLR-4, anti-MHC-II, anti-CD80 and anti-CD40. The dot plot and bar charts are representative of three independent experiments; * $p < 0.05$.

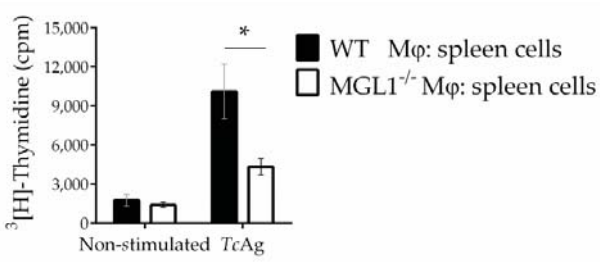


Figure 5. MGL1$^{-/-}$ PE-Mϕ induce a deficient activation of *T. cruzi* antigen-specific lymphocytes. PE-Mϕ from MGL1$^{-/-}$ or WT mice were stimulated with TcAg, two hours later Mϕ were washed to remove the non-phagocytosed antigen. Splenocytes from 21 days-infected WT mice were added at ratio of 1:10. After 5 days, cell proliferation was assessed by [^{3}H]thymidine incorporation. Data are representative of three separate experiments and are plotted as the means of triplicate wells (± SEM), n = 7, * $p < 0.05$.

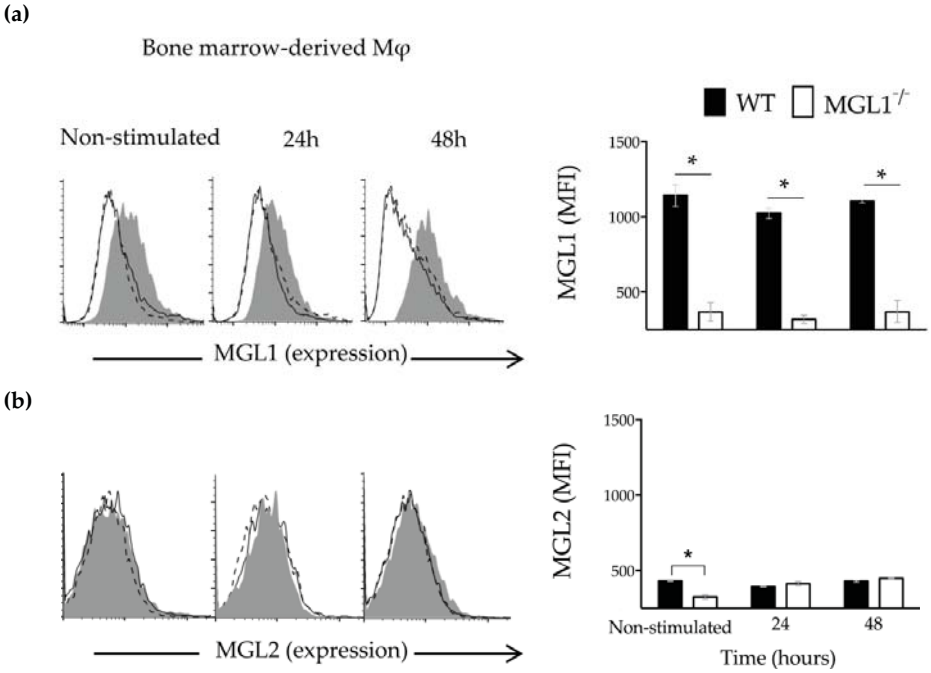


Figure 6. Expression of MGL1 and MGL2 in BMMϕ in response to *TcAg*. BMMϕ (F4/80+) from WT or MGL1$^{-/-}$ mice were stimulated for 24 or 48 h with *Tc*Ag (25 μg/mL). Representative histogram and bar chart of the percentage of BMMϕ expressing MGL1 and MGL2 are shown in (**a**) and (**b**), respectively. Dotted line, isotype; gray area, Mϕ from WT mice; solid line, Mϕ from MGL1$^{-/-}$ mice; n = 6 mice per group; and * $p < 0.05$.

Next, the MGL1 and MGL2 expression was examined using BMMϕ from MGL1$^{-/-}$ mice. As show in Figure 6, the absence of expression of MGL1 and MGL2 was confirmed on BMMϕ from MGL1$^{-/-}$ mice at baseline (Figure 6a,b; MGL1$^{-/-}$ Mϕ, non-stimulated, solid line). After *Tc*Ag stimulation, MGL1$^{-/-}$ BMMϕ did not show expression of MGL1, neither MGL2 (Figure 1a,b; MGL1$^{-/-}$ Mϕ, solid line). These results demonstrate that BMMϕ from MGL1$^{-/-}$ mice are unable to upregulate MGL2 in response to *Tc*Ag, suggesting that there is not a compensatory hyperexpression of this CLR in the absence of MGL1.

3.6. MGL1 Deficiency in BMMϕ Results in Reduced Activation of the NFκ-B and ERK1/2 Signaling Pathways in Response to T. cruzi Antigen

The activation of Mϕ by infectious and non-infectious insults is usually triggered by the recognition of the antigen by receptors on the surface of Mϕ; this leads to the activation of the NFκ-B and c-Jun transcription factors, which play key roles in modulating the expression of many proinflammatory genes and innate immune response signaling [32–34]. Consequently, IL-10, TNF-α, and NO production by Mϕ correlates with the strength of NFκ-B activation. Moreover, it is well established that the MAPK cascade also controls the post-transcriptional regulation of TNF-α [35]; the kinases ERK and P38 are notable members of the MAPK family. Therefore, to investigate the role of MGL1 in the activation of ERK1/2, p38, c-Jun and NFκ-B; WT and MGL1$^{-/-}$ BMMϕ were stimulated for 5, 15 and 30 min with LPS or *Tc*Ag, and the phosphorylation of these proteins was measured.

MGL1$^{-/-}$ Mϕ showed a significant reduction in the phosphorylation of ERK1, 2, c-Jun and NFκ-B after LPS or *Tc*Ag stimulation compared with that of WT Mϕ. In contrast, comparable levels of phosphorylated p38 were observed between WT and MGL1$^{-/-}$ Mϕ (Figure 7a,b). These results suggest that the MGL1 may be signaling trough the ERK1/2 c-Jun and NFκ-B axis in response to *Tc*Ag, but not p38, thus MGL1 is critical for the optimal activation of Mϕ during *T. cruzi* infection and consequently favor secretion of proinflammatory cytokines.

3.7. MGL1 Regulates the Expression of the NLP3 Sensor

One of the main factors that trigger acute or chronic inflammation is the inflammasome [36]. Assembly of PRRs-mediated inflammasomes, such as the NLRP3 sensor, one of the most important inflammasomes involved in the development of the inflammatory microenvironment through the activation of caspase-1, results in the secretion of IL-1β, which ultimately creates an inflammatory microenvironment [37]. Because MGL1$^{-/-}$ Mϕ exposed to LPS or *Tc*Ag exhibited decreased production of proinflammatory cytokines, which also correlated with deficient ERK1/2, c-Jun and NFκ-B activation, we explored whether MGL1$^{-/-}$ Mϕ may display deficient expression of the NLRP3 receptor. WT and MGL1$^{-/-}$ BMMϕ were stimulated with LPS, *Tc*Ag or LPS/*Tc*Ag for 24 h. After incubation, total protein was extracted, and western blot analysis was performed to quantify protein expression levels of NLRP3, procaspase-1 and caspase-1.

As shown in Figure 8a,b; WT BMMϕ stimulated with LPS, *Tc*Ag or LPS/*Tc*Ag displayed increased NLRP3 protein expression levels compared to non-stimulated cells. Importantly, compared to WT BMMϕ, MGL1$^{-/-}$ BMMϕ did not show any increase in NLRP3 protein levels neither procaspase-1, or caspase-1 upon stimulation with LPS/*Tc*Ag or either stimulus alone. Interestingly, MGL1$^{-/-}$ BMMϕ also displayed reduced IL-1β production in response to LPS, *Tc*Ag or LPS/*Tc*Ag compared to those levels found in WT BMMϕ exposed to the same stimuli.

Cells **2020**, *9*, 108

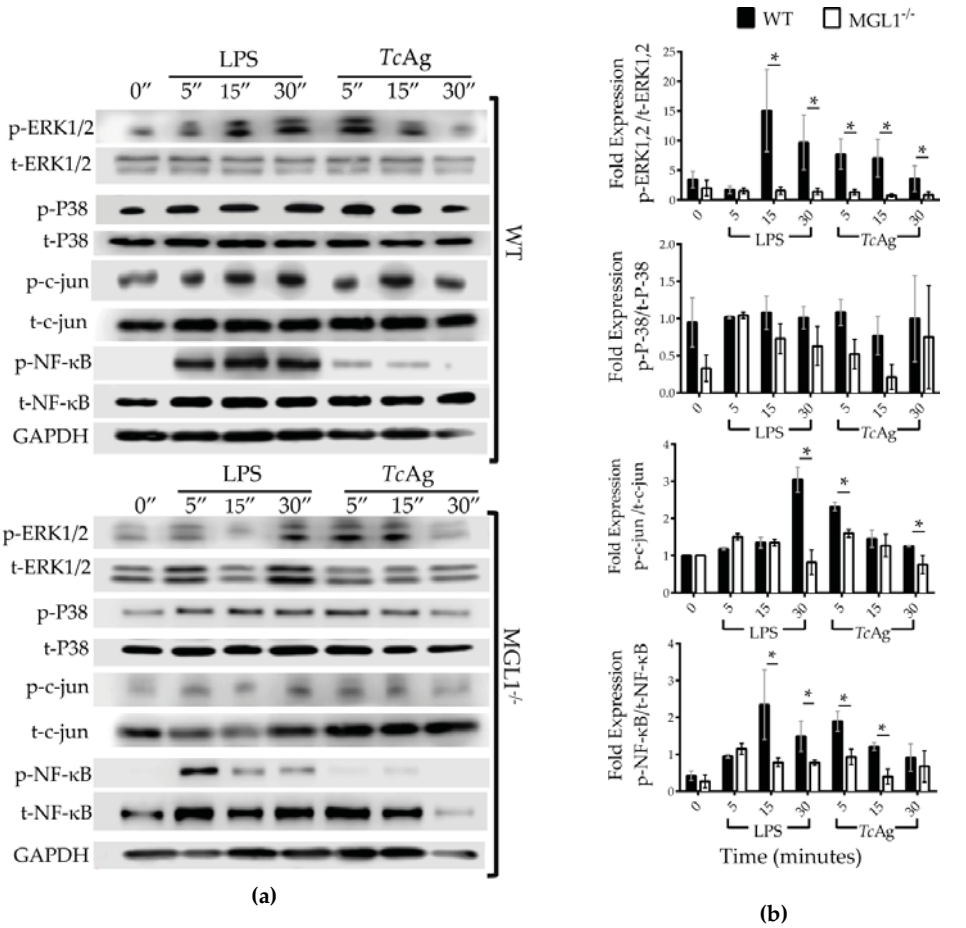


Figure 7. MGL1$^{-/-}$ BMMϕ exhibit reduced activation of ERK1/2, c-Jun and NFκ-B signaling pathway in response to *Tc*Ag. (**a**) WT and MGL1$^{-/-}$ BMMϕ were stimulated for 5, 15 and 30 min with LPS (100 ng/mL) or *Tc*Ag (25 μg/mL). In total protein lysates of Mϕ were measured the phosphorylation of ERK1/2, p38 and NFκB by western blot analysis. Western blotting for the indicated proteins. (**b**) Densitometry analysis for the indicated proteins as described above. Results are representative of at least three separate experiments each with three biological replicates. Densitometry analysis of the indicated proteins was normalized first, to GAPDH protein, and next to total protein expression as appropriate. Data shown are mean ± SEM (n = 6), * $p < 0.05$.

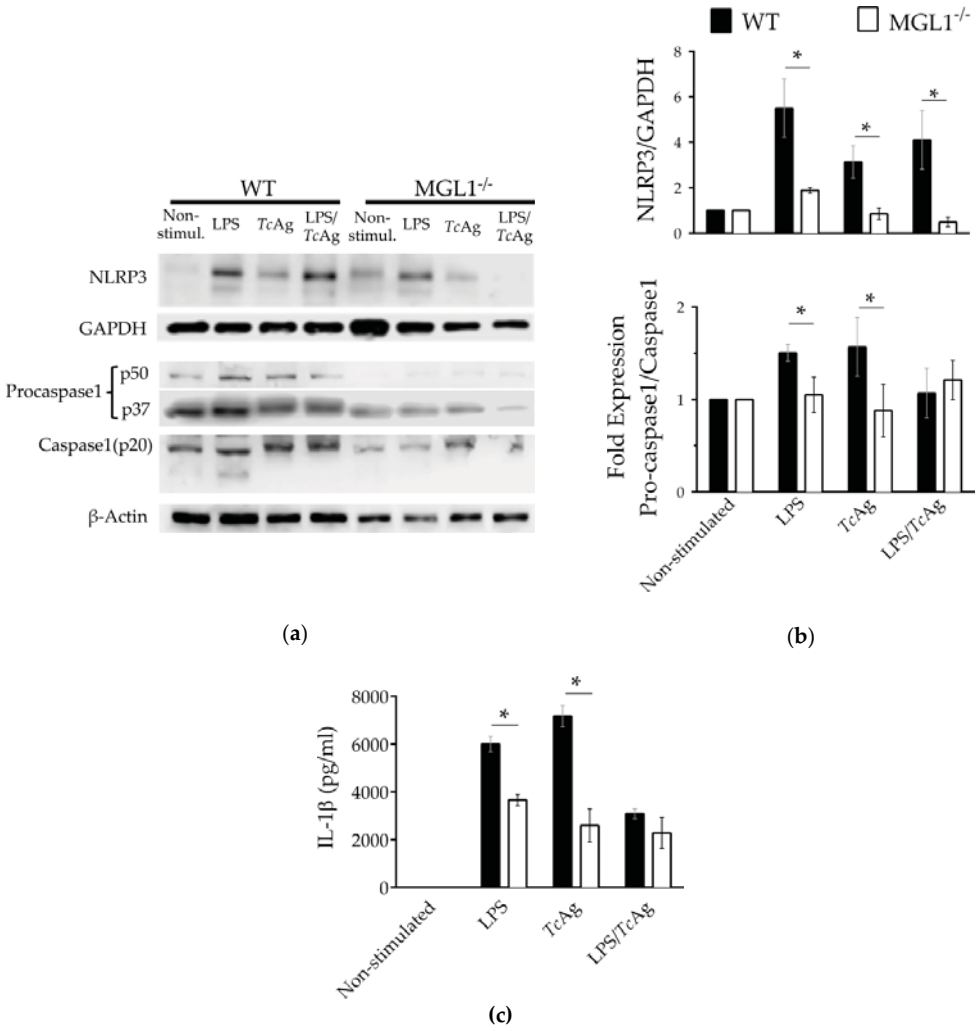


Figure 8. MGL1 is required for proper expression of NLRP3, pro-caspase1/caspase1 and IL-1β production. WT and MGL1$^{-/-}$ BMMϕ were stimulated for 24 h with LPS (100 ng/mL) or *Tc*Ag (25 μg/mL) or LPS+*Tc*Ag (100 ng + 25 μg/ mL), in total protein lysates of Mϕ were measured NLRP3, pro-caspase1 and caspase1 by western blot. (**a**) Western blot showing down-regulated NLRP3, pro-caspase1 and caspase1 protein expression in MGL1$^{-/-}$ Mϕ, and (**b**) the densitometry analysis for the NLRP3 and the fold expression of pro-caspase1/caspase 1. (**c**) ELISA quantification of IL-1β in the cell culture supernatants. The western blot data were normalized to GAPDH control and are representative of two separate experiments. The data are plotted as the means (± SEM), n = 5, * $p < 0.05$.

4. Discussion

Recent evidence suggests that MGL plays an important role that goes beyond the sensing and elimination of dead and dying cells [38]. For example, MGL is able to recognize abnormal glycosylation patterns, a common characteristic of malignant cells, which allows the interaction of cancer cells with platelets, leukocytes and endothelial cells, facilitating tumor invasion, metastasis and the immunosuppressive response [39]. Therefore, GalNAc-carrying tumor-associated antigens or anti-MGL antibodies have been used as ligands to identify the role of MGL in the activation of DCs in vitro [19,31,40–42].

The generation of MGL1$^{-/-}$ mice allows for investigating the immunological functions of MGL1 separately from that of MGL2 at the cellular level; using these mice, it has been possible to establish that MGL1 is capable of recognize glycosylated structures expressed in parasites including protozoa and helminths [8,22]. Our previous studies, conducted in a mouse model, demonstrated that the interaction of MGL1 with highly glycosylated structures of *T. cruzi* (as a natural ligand) plays an essential role in immunity by increasing Mϕ activation and parasite killing during in vivo *T. cruzi* infection [22,43]. Despite this evidence pointing to a role for MGL1 in the immune response, how MGL1 modulates the immune response against this pathogen remains unclear. In the current study we demonstrated that in PE-Mϕ and BMMϕ from MGL1$^{-/-}$ mice, MGL1 is totally absent, while the MGL2 expression is very low or absent, respectively. Thus, using MGL1$^{-/-}$ BMMϕ, we provide important insights into the mechanism by which the MGL1-*Tc*Ag interaction activates Mϕ against *T. cruzi* infection.

Having demonstrated that MGL1$^{-/-}$ Mϕ infected in vitro with *T. cruzi* presented greater numbers of internalized parasites than WT Mϕ [22], we extended this observation by showing that internalized parasites in MGL1$^{-/-}$ Mϕ remain alive. Moreover, following overnight incubation of MGL1$^{-/-}$ Mϕ with IFN-γ, LPS or both, there was not a significant reduction in the number of intracellular parasites, as occurred in the WT Mϕ. This activation deficiency of MGL1$^{-/-}$ Mϕ was accompanied by a remarkable downregulation of ROS and NO production, as well as by a reduction in the levels of IL-12 and TNF-α, which are important for controlling parasite infection and replication [44,45]. These observations suggest that MGL1 may recognize *T. cruzi* and favors a cross-talk between MGL1 signalling pathway and other important pathways for Mϕ activation that helps to activate the oxidative burst.

We previously observed that MGL1$^{-/-}$ Mϕ infected in vitro with trypomastigotes of *T. cruzi* (extracellular and infective blood form) had a negatively regulated expression of MHC-II and TLR4 [22]. We asked whether this phenomenon also occurred in MGL1$^{-/-}$ Mϕ treated with *Tc*Ag or epimastigotes of *T. cruzi* (a form of transition found in crops and in the insect vector). We decided to expose Mϕ to culture-derived epimastigotes and not to blood trypomastigotes due to the fact that this later stage could be opsonized and major antigens neutralized [46]. Moreover, *T. cruzi* epimastigotes share GPI-anchored antigens with trypomastigotes [47], and we have previously shown that epimastigotes express MGL1 ligands (Galactose and N-Acetylgalactosamine) as revealed by staining with the lectin Jacalin [22]. Here, we demonstrate that MGL1$^{-/-}$ Mϕ exposed to *Tc*Ag or live epimastigotes of *T. cruzi* displayed downregulated expression of TLR4, MHC-II and CD40, but not CD80 expression, which correlated with a deficiency in *Tc*Ag presentation to activate antigen-specific T cells. This finding is consistent with a previous report by Napoletano et al., who showed that the interaction of MGL with tumor-associated antigens or an anti-MGL antibody in DCs improved the performance of DCs as antigen-presenting cells, promoting the positive regulation of markers of maturation and increasing the activation of antigen-specific CD8 T cells [41]. In contrast, van Vliet et al. observed that anti-MGL antibody treatment combined with TLR stimulation did not affect the expression of CD80, CD83, CD86 or MHC-II [31]. This discrepancy may suggest that the expression of costimulatory molecules, driven by MGL, is different depending on the stimulus. For instance, the MGL1$^{-/-}$ PE-Mϕ infected with the fungi *C. albicans* displayed similar ROS production compared to WT PE-Mϕ, indicating a MGL1-independent recognition of this pathogen as well as an efficient oxidative burst. In line with this idea, our previous report demonstrated that MGL1$^{-/-}$PE-Mϕ exposed to POLY: IC, a TLR-4-independent stimulus, induced high levels of NO and inflammatory cytokines [22]; indicating that Mϕ activation is intact if the insult is independent of the interaction with MGL1, TLR4 or both.

T. cruzi parasites have abundant amounts of glycosylated molecules such as GPI- and GIPL-mucins, which are potent activators of TLR-2 and TLR-4, respectively [48]. Although Mϕ stimulated with GPI-mucin were found to produce TNF-α, IL-12 and NO via TLR-2 [49], Mϕ from TLR-2$^{-/-}$ mice displayed partial phosphorylation of ERK1/2, detectable levels of TNF-α and reactive nitrogen intermediate production, compared to the completely unresponsive Myd88$^{-/-}$ (myeloid differentiation factor 88; an essential signal transducer for TLRs) Mϕ, indicating that *T. cruzi* parasites activate an alternative inflammatory pathway independent of TLR-2 [44]. Monocytes stimulated with GIPL-mucin

induce NFκ-B activation via TLR-4, whereas TLR-4/MD-2 CHO cells (TLR-4 non-functional monocytes) do not [50]. Importantly, in DCs the costimulation of MGL with agonistic antibodies or carbohydrate ligands augments TLR-2-mediated responses, favoring IL-10 and TNF-α secretion [31]. Here, we showed that MGL1$^{-/-}$ Mϕ infected in vitro with epimastigotes of *T. cruzi* or exposed to *Tc*Ag showed reduced expression of TLR-2 and TLR-4, consequently, MGL1$^{-/-}$ Mϕ displayed a poor response to LPS. This support that MGL1, in coordination with TLR-4, is involved in *T. cruzi* recognition and Mϕ activation, which could explain the defective production of TNF-α and IL-12, as well as the reduced ROS and NO production observed in MGL1$^{-/-}$ Mϕ.

Furthermore, we found reduced phosphorylation of ERK1/2 and NFκ-B in MGL1$^{-/-}$ Mϕ stimulated with *Tc*Ag compared to similarly exposed WT Mϕ. These observations are consistent with previous reports demonstrating that MGL engagement with GalNac-carrying tumor-associated antigens or anti-MGL antibodies induced the phosphorylation of ERK1/2, c-Jun and NFκ-B activation in human DCs [31,41]. Thus, our results reveal that the possible recognition of *T. cruzi* by MGL1 activates intracellular signaling cascades that modulate the innate immune response, and this role requires the simultaneous activation of TLR molecules for its immune effects.

Although it is well known that TLRs profoundly influence the Mϕ inflammatory response, this is not the only way by which the inflammatory response can be activated. Studies have shown that, through both direct and indirect mechanisms, CLRs such as Galectin-3 and Dectin-1 can activate the NLRP3, NLRC4 or caspase-8 inflammasomes, leading to the production of IL-1β [51,52]. Our study provides evidence for the first time that the MGL1 receptor contributes to NLRP3 inflammasome expression in response to both LPS and *Tc*Ag.

Inflammasome activation is crucial for host defence in the acute phase of *T. cruzi* infection. In a previous report, mice lacking NLRP3 or caspase-12 genes exhibited increased numbers of *T. cruzi* parasites [53]. Moreover, *Tc*Ag or LPS increase NLRP3 activation in Mϕ from WT mice [54]. However, we observed that MGL1$^{-/-}$ Mϕ had a diminished ability to produce IL-1β most likely as a result of the impaired inflammasome activation, in response to LPS and *Tc*Ag. In *T. cruzi* infection, the processing of IL-1β into its bioactive form requires two stimuli: first, the recognition of the pathogen that induces pro-IL-1β gene transcription, then ROS elicited by *T. cruzi* infection serve as the second signal for caspase-1 induction that triggers pro-IL-1β cleavage to active IL-1β [54,55]. It seems that the lack of interaction between *Tc*Ag and MGL1 in our model inhibits the expression of the inflammasome NLRP3, consequently, also the canonical pathway of caspase-1. This suggests that overall the inflammasome machinery is dampened in MGL1-deficient Mϕ, probably because *T. cruzi* is not fully recognized in the absence of MGL1. We detected some IL-1β, even in the absence of ROS, suggesting that there is a compensatory mechanism or NLRP3-independent release of IL-1β bioactive, which could be an interesting topic for a future study. Whether the non-canonical (caspase 11-mediated) NLRP3 inflammasome pathway is altered in MGL1$^{-/-}$ Mϕ remains to be confirmed. Our findings are in line with those previously reported showing that other lectins, such as galectin-3, are able to trigger NLRP3 activation in the context of liver diseases and influenza infection [52,56].

Based on the results generated in this work, we propose a hypothetical model to explain the role of MGL1 in Mϕ activation, in response to *T. cruzi* infection (Figure 9).

induce NFκ-B activation via TLR-4, whereas TLR-4/MD-2 CHO cells (TLR-4 non-functional monocytes) do not [50]. Importantly, in DCs the costimulation of MGL with agonistic antibodies or carbohydrate ligands augments TLR-2-mediated responses, favoring IL-10 and TNF-α secretion [31]. Here, we showed that MGL1$^{-/-}$ Mϕ infected in vitro with epimastigotes of *T. cruzi* or exposed to *Tc*Ag showed reduced expression of TLR-2 and TLR-4, consequently, MGL1$^{-/-}$ Mϕ displayed a poor response to LPS. This support that MGL1, in coordination with TLR-4, is involved in *T. cruzi* recognition and Mϕ activation, which could explain the defective production of TNF-α and IL-12, as well as the reduced ROS and NO production observed in MGL1$^{-/-}$ Mϕ.

Furthermore, we found reduced phosphorylation of ERK1/2 and NFκ-B in MGL1$^{-/-}$ Mϕ stimulated with *Tc*Ag compared to similarly exposed WT Mϕ. These observations are consistent with previous reports demonstrating that MGL engagement with GalNac-carrying tumor-associated antigens or anti-MGL antibodies induced the phosphorylation of ERK1/2, c-Jun and NFκ-B activation in human DCs [31,41]. Thus, our results reveal that the possible recognition of *T. cruzi* by MGL1 activates intracellular signaling cascades that modulate the innate immune response, and this role requires the simultaneous activation of TLR molecules for its immune effects.

Although it is well known that TLRs profoundly influence the Mϕ inflammatory response, this is not the only way by which the inflammatory response can be activated. Studies have shown that, through both direct and indirect mechanisms, CLRs such as Galectin-3 and Dectin-1 can activate the NLRP3, NLRC4 or caspase-8 inflammasomes, leading to the production of IL-1β [51,52]. Our study provides evidence for the first time that the MGL1 receptor contributes to NLRP3 inflammasome expression in response to both LPS and *Tc*Ag.

Inflammasome activation is crucial for host defence in the acute phase of *T. cruzi* infection. In a previous report, mice lacking NLRP3 or caspase-12 genes exhibited increased numbers of *T. cruzi* parasites [53]. Moreover, *Tc*Ag or LPS increase NLRP3 activation in Mϕ from WT mice [54]. However, we observed that MGL1$^{-/-}$ Mϕ had a diminished ability to produce IL-1β most likely as a result of the impaired inflammasome activation, in response to LPS and *Tc*Ag. In *T. cruzi* infection, the processing of IL-1β into its bioactive form requires two stimuli: first, the recognition of the pathogen that induces pro-IL-1β gene transcription, then ROS elicited by *T. cruzi* infection serve as the second signal for caspase-1 induction that triggers pro-IL-1β cleavage to active IL-1β [54,55]. It seems that the lack of interaction between *Tc*Ag and MGL1 in our model inhibits the expression of the inflammasome NLRP3, consequently, also the canonical pathway of caspase-1. This suggests that overall the inflammasome machinery is dampened in MGL1-deficient Mϕ, probably because *T. cruzi* is not fully recognized in the absence of MGL1. We detected some IL-1β, even in the absence of ROS, suggesting that there is a compensatory mechanism or NLRP3-independent release of IL-1β bioactive, which could be an interesting topic for a future study. Whether the non-canonical (caspase 11-mediated) NLRP3 inflammasome pathway is altered in MGL1$^{-/-}$ Mϕ remains to be confirmed. Our findings are in line with those previously reported showing that other lectins, such as galectin-3, are able to trigger NLRP3 activation in the context of liver diseases and influenza infection [52,56].

Based on the results generated in this work, we propose a hypothetical model to explain the role of MGL1 in Mϕ activation, in response to *T. cruzi* infection (Figure 9).

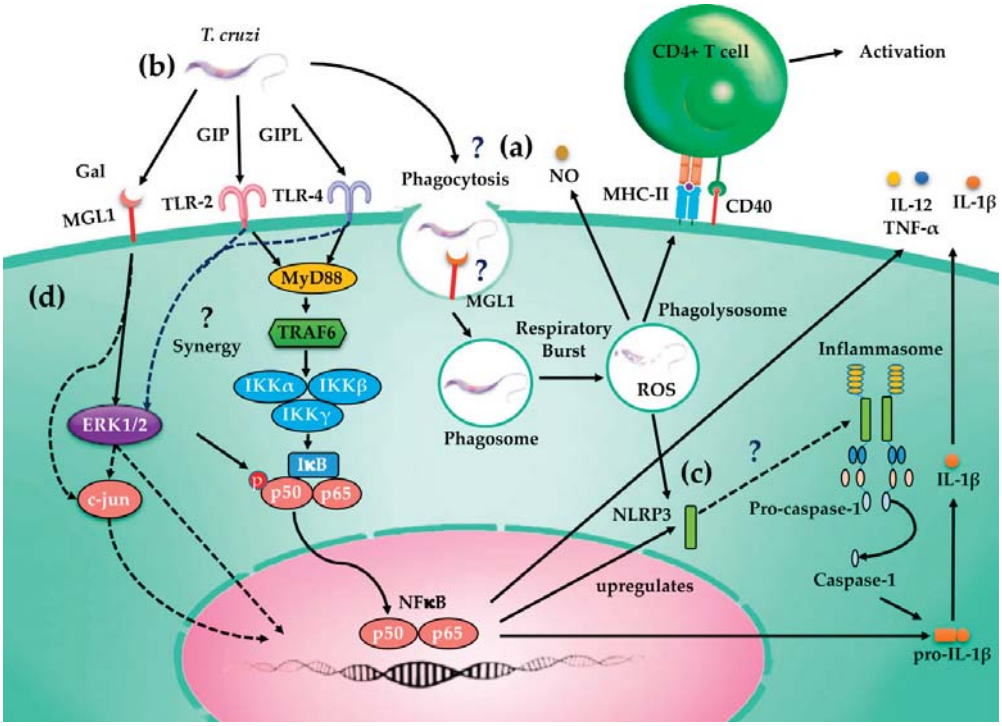


Figure 9. Functions of MGL1 in macrophage response to *T. cruzi* infection. (**a**) MGL1 recognizes glycosylated molecules and may mediate the uptake of *T. cruzi* through phagocytosis, inducing antimicrobial effector mechanisms such as respiratory burst and NO production. This leads to the induction of MHC-II and co-stimulatory molecules and increased antigen presentation to CD4+T cells. (**b**) The recognition of extracellular *T. cruzi* by MGL1 in Mϕ induces intracellular signaling through the activation of ERK1/2 and NFκB, as well as the expression of TLR2 and TLR4, that result in the transcription of proinflammatory cytokines, such as TNF-α and IL-12, although anti-inflammatory cytokines such as IL-10 are also produced. (**c**) Moreover, MGL1 appears to favor the active form of the NLPR3 inflammasome; consequently, the assembled of pro-caspase 1, which in turn, by autoproteolysis, generates caspase 1 responsible for the cleavage pro-IL-1β to its active form of IL-1β. d) Dashed lines indicate incomplete understood mechanisms.

5. Conclusions

In conclusion, here, we showed that MGL1 engagement with *Tc*Ag enables Mϕ to perform APC functions and provide additional evidence to support the hypothesis that MGL1 can upregulate Mϕ activation. Another novel finding was that MGL1 synergizes with TLR-2 and TLR-4 to upregulate ERK1/2, c-Jun, NFκ-B-dependent expression of proinflammatory factors. Furthermore, we provided the first evidence that MGL1 contributes to the innate immune response via the NLRP3 inflammasome expression and ROS production in response to *Tc*Ag and LPS. Finally, our data offer insight into the mechanisms involved in controlling *T. cruzi* infection by MGL1, although more details of this early response have yet to be described.

Author Contributions: Conceptualization, M.R.-S., and L.I.T.; methodology and investigation, T.R., I.J.-A., O.N.-Y. and A.V.-M.; data curation, T.R. and M.R.-S.; writing—original draft preparation, T.R.; writing—review and editing. T.P.-F., J.L.R. and M.R.-S.; resources, J.L.R. and L.I.T.; supervision A.V.-M. and L.I.T.; funding acquisition, M.R.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part, by the Support Program for Research Projects and Technological Innovation" (PAPIIT)-UNAM, grant number IN-209718 and by the National Council of Science and Technology of Mexico (CONACYT), grant number A1-S-10463.

Acknowledgments: This work is part of the requirements to obtain a PhD degree in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) for Tonathiu Rodriguez (CVU CONACyT No. 5502200). Tonathiu Rodriguez and Thalia Pacheco received a fellowship from the National Council of Science and Technology of Mexico (CONACyT).

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

References

1. Zelensky, A.N.; Gready, J.E. The C-type lectin-like domain superfamily. *FEBS J.* **2005**, *272*, 6179–6217. [CrossRef] [PubMed]
2. Vázquez-Mendoza, A.; Carrero, J.C.; Rodriguez-Sosa, M. Parasitic infections: A role for C-type lectins receptors. *Biomed. Res. Int.* **2013**, *2013*, 11. [CrossRef] [PubMed]
3. Redelinghuys, P.; Brown, G.D. Inhibitory C-type lectin receptors in myeloid cells. *Immunol. Lett.* **2011**, *136*, 1–12. [CrossRef] [PubMed]
4. Unger, W.W.; van Beelen, A.J.; Bruijns, S.C.; Joshi, M.; Fehres, C.M.; van Bloois, L.; Verstege, M.I.; Ambrosini, M.; Kalay, H.; Nazmi, K. Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells. *J. Control. Release* **2012**, *160*, 88–95. [CrossRef] [PubMed]
5. Kerrigan, A.M.; Brown, G.D. C-type lectins and phagocytosis. *Immunobiology* **2009**, *214*, 562–575. [CrossRef] [PubMed]
6. Graham, L.M.; Brown, G.D. The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine* **2009**, *48*, 148–155. [CrossRef] [PubMed]
7. Brown, G.D.; Willment, J.A.; Whitehead, L. C-type lectins in immunity and homeostasis. *Nat. Rev. Immunol.* **2018**, *18*, 374–389. [CrossRef]
8. Montero-Barrera, D.; Valderrama-Carvajal, H.; Terrazas, C.A.; Rojas-Hernandez, S.; Ledesma-Soto, Y.; Vera-Arias, L.; Carrasco-Yepez, M.; Gomez-Garcia, L.; Martinez-Saucedo, D.; Becerra-Diaz, M.; et al. The macrophage galactose-type lectin-1 (MGL1) recognizes Taenia crassiceps antigens, triggers intracellular signaling, and is critical for resistance to this infection. *Biomed. Res. Int.* **2015**, *2015*, 615865. [CrossRef]
9. Del Fresno, C.; Iborra, S.; Saz-Leal, P.; Martinez-Lopez, M.; Sancho, D. Flexible Signaling of Myeloid C-Type Lectin Receptors in Immunity and Inflammation. *Front. Immunol.* **2018**, *9*, 804. [CrossRef]
10. Brubaker, S.W.; Bonham, K.S.; Zanoni, I.; Kagan, J.C. Innate immune pattern recognition: A cell biological perspective. *Annu. Rev. Immunol.* **2015**, *33*, 257–290. [CrossRef]
11. van Kooyk, Y. C-type lectins on dendritic cells: Key modulators for the induction of immune responses. *Biochem. Soc. Trans.* **2008**, *36*, 1478–1481. [CrossRef] [PubMed]
12. Lefèvre, L.; Lugo-Villarino, G.; Meunier, E.; Valentin, A.; Olagnier, D.; Authier, H.; Duval, C.; Dardenne, C.; Bernad, J.; Lemesre, J.L. The C-type lectin receptors dectin-1, MR, and SIGNR3 contribute both positively and negatively to the macrophage response to Leishmania infantum. *Immunity* **2013**, *38*, 1038–1049. [CrossRef] [PubMed]
13. Ward, S.E.; O'Sullivan, J.M.; Drakeford, C.; Aguila, S.; Jondle, C.N.; Sharma, J.; Fallon, P.G.; Brophy, T.M.; Preston, R.J.S.; Smyth, P.; et al. A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance. *Blood* **2018**, *131*, 911–916. [CrossRef] [PubMed]
14. Sato, M.; Kawakami, K.; Osawa, T.; Toyoshima, S. Molecular cloning and expression of cDNA encoding a galactose/N-acetylgalactosamine-specific lectin on mouse tumoricidal macrophages. *J. Biochem.* **1992**, *111*, 331–336. [CrossRef]
15. Tsuiji, M.; Fujimori, M.; Ohashi, Y.; Higashi, N.; Onami, T.M.; Hedrick, S.M.; Irimura, T. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. *J. Biol. Chem.* **2002**, *277*, 28892–28901. [CrossRef]
16. van Vliet, S.J.; Aarnoudse, C.A.; Broks-van den Berg, V.C.; Boks, M.; Geijtenbeek, T.B.; van Kooyk, Y. MGL-mediated internalization and antigen presentation by dendritic cells: A role for tyrosine-5. *Eur. J. Immunol.* **2007**, *37*, 2075–2081. [CrossRef]

Funding: This research was funded in part, by the Support Program for Research Projects and Technological Innovation" (PAPIIT)-UNAM, grant number IN-209718 and by the National Council of Science and Technology of Mexico (CONACYT), grant number A1-S-10463.

Acknowledgments: This work is part of the requirements to obtain a PhD degree in the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) for Tonathiu Rodriguez (CVU CONACyT No. 5502200). Tonathiu Rodriguez and Thalia Pacheco received a fellowship from the National Council of Science and Technology of Mexico (CONACyT).

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

References

1. Zelensky, A.N.; Gready, J.E. The C-type lectin-like domain superfamily. *FEBS J.* **2005**, *272*, 6179–6217. [CrossRef] [PubMed]
2. Vázquez-Mendoza, A.; Carrero, J.C.; Rodriguez-Sosa, M. Parasitic infections: A role for C-type lectins receptors. *Biomed. Res. Int.* **2013**, *2013*, 11. [CrossRef] [PubMed]
3. Redelinghuys, P.; Brown, G.D. Inhibitory C-type lectin receptors in myeloid cells. *Immunol. Lett.* **2011**, *136*, 1–12. [CrossRef] [PubMed]
4. Unger, W.W.; van Beelen, A.J.; Bruijns, S.C.; Joshi, M.; Fehres, C.M.; van Bloois, L.; Verstege, M.I.; Ambrosini, M.; Kalay, H.; Nazmi, K. Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells. *J. Control. Release* **2012**, *160*, 88–95. [CrossRef] [PubMed]
5. Kerrigan, A.M.; Brown, G.D. C-type lectins and phagocytosis. *Immunobiology* **2009**, *214*, 562–575. [CrossRef] [PubMed]
6. Graham, L.M.; Brown, G.D. The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine* **2009**, *48*, 148–155. [CrossRef] [PubMed]
7. Brown, G.D.; Willment, J.A.; Whitehead, L. C-type lectins in immunity and homeostasis. *Nat. Rev. Immunol.* **2018**, *18*, 374–389. [CrossRef]
8. Montero-Barrera, D.; Valderrama-Carvajal, H.; Terrazas, C.A.; Rojas-Hernandez, S.; Ledesma-Soto, Y.; Vera-Arias, L.; Carrasco-Yepez, M.; Gomez-Garcia, L.; Martinez-Saucedo, D.; Becerra-Diaz, M.; et al. The macrophage galactose-type lectin-1 (MGL1) recognizes Taenia crassiceps antigens, triggers intracellular signaling, and is critical for resistance to this infection. *Biomed. Res. Int.* **2015**, *2015*, 615865. [CrossRef]
9. Del Fresno, C.; Iborra, S.; Saz-Leal, P.; Martinez-Lopez, M.; Sancho, D. Flexible Signaling of Myeloid C-Type Lectin Receptors in Immunity and Inflammation. *Front. Immunol.* **2018**, *9*, 804. [CrossRef]
10. Brubaker, S.W.; Bonham, K.S.; Zanoni, I.; Kagan, J.C. Innate immune pattern recognition: A cell biological perspective. *Annu. Rev. Immunol.* **2015**, *33*, 257–290. [CrossRef]
11. van Kooyk, Y. C-type lectins on dendritic cells: Key modulators for the induction of immune responses. *Biochem. Soc. Trans.* **2008**, *36*, 1478–1481. [CrossRef] [PubMed]
12. Lefèvre, L.; Lugo-Villarino, G.; Meunier, E.; Valentin, A.; Olagnier, D.; Authier, H.; Duval, C.; Dardenne, C.; Bernad, J.; Lemesre, J.L. The C-type lectin receptors dectin-1, MR, and SIGNR3 contribute both positively and negatively to the macrophage response to Leishmania infantum. *Immunity* **2013**, *38*, 1038–1049. [CrossRef] [PubMed]
13. Ward, S.E.; O'Sullivan, J.M.; Drakeford, C.; Aguila, S.; Jondle, C.N.; Sharma, J.; Fallon, P.G.; Brophy, T.M.; Preston, R.J.S.; Smyth, P.; et al. A novel role for the macrophage galactose-type lectin receptor in mediating von Willebrand factor clearance. *Blood* **2018**, *131*, 911–916. [CrossRef] [PubMed]
14. Sato, M.; Kawakami, K.; Osawa, T.; Toyoshima, S. Molecular cloning and expression of cDNA encoding a galactose/N-acetylgalactosamine-specific lectin on mouse tumoricidal macrophages. *J. Biochem.* **1992**, *111*, 331–336. [CrossRef]
15. Tsuiji, M.; Fujimori, M.; Ohashi, Y.; Higashi, N.; Onami, T.M.; Hedrick, S.M.; Irimura, T. Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1. *J. Biol. Chem.* **2002**, *277*, 28892–28901. [CrossRef]
16. van Vliet, S.J.; Aarnoudse, C.A.; Broks-van den Berg, V.C.; Boks, M.; Geijtenbeek, T.B.; van Kooyk, Y. MGL-mediated internalization and antigen presentation by dendritic cells: A role for tyrosine-5. *Eur. J. Immunol.* **2007**, *37*, 2075–2081. [CrossRef]

17. Upham, J.P.; Pickett, D.; Irimura, T.; Anders, E.M.; Reading, P.C. Macrophage receptors for influenza A virus: Role of the macrophage galactose-type lectin and mannose receptor in viral entry. *J. Virol.* **2010**, *84*, 3730–3737. [CrossRef]
18. Singh, S.K.; Streng-Ouwehand, I.; Litjens, M.; Weelij, D.R.; Garcia-Vallejo, J.J.; van Vliet, S.J.; Saeland, E.; van Kooyk, Y. Characterization of murine MGL1 and MGL2 C-type lectins: Distinct glycan specificities and tumor binding properties. *Mol. Immunol.* **2009**, *46*, 1240–1249. [CrossRef]
19. Denda-Nagai, K.; Aida, S.; Saba, K.; Suzuki, K.; Moriyama, S.; Oo-Puthinan, S.; Tsuiji, M.; Morikawa, A.; Kumamoto, Y.; Sugiura, D.; et al. Distribution and function of macrophage galactose-type C-type lectin 2 (MGL2/CD301b): Efficient uptake and presentation of glycosylated antigens by dendritic cells. *J. Biol. Chem.* **2010**, *285*, 19193–19204. [CrossRef]
20. Filardy, A.A.; Guimaraes-Pinto, K.; Nunes, M.P.; Zukeram, K.; Fliess, L.; Pereira, L.; Oliveira Nascimento, D.; Conde, L.; Morrot, A. Human Kinetoplastid Protozoan Infections: Where Are We Going Next? *Front. Immunol.* **2018**, *9*, 1493. [CrossRef]
21. Acosta-Serrano, A.; Almeida, I.C.; Freitas-Junior, L.H.; Yoshida, N.; Schenkman, S. The mucin-like glycoprotein super-family of Trypanosoma cruzi: Structure and biological roles. *Mol. Biochem. Parasitol.* **2001**, *114*, 143–150. [CrossRef]
22. Vazquez, A.; Ruiz-Rosado Jde, D.; Terrazas, L.I.; Juarez, I.; Gomez-Garcia, L.; Calleja, E.; Camacho, G.; Chavez, A.; Romero, M.; Rodriguez, T.; et al. Mouse macrophage galactose-type lectin (mMGL) is critical for host resistance against Trypanosoma cruzi infection. *Int. J. Biol. Sci.* **2014**, *10*, 909–920. [CrossRef]
23. Onami, T.M.; Lin, M.Y.; Page, D.M.; Reynolds, S.A.; Katayama, C.D.; Marth, J.D.; Irimura, T.; Varki, A.; Varki, N.; Hedrick, S.M. Generation of mice deficient for macrophage galactose- and N-acetylgalactosamine-specific lectin: Limited role in lymphoid and erythroid homeostasis and evidence for multiple lectins. *Mol. Cell. Biol.* **2002**, *22*, 5173–5181. [CrossRef]
24. Laird, P.W.; Zijderveld, A.; Linders, K.; Rudnicki, M.A.; Jaenisch, R.; Berns, A. Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* **1991**, *19*, 4293. [CrossRef]
25. Migliorini, P.; Corradin, G.; Corradin, S.B. Macrophage NO2-production as a sensitive and rapid assay for the quantitation of murine IFN-gamma. *J. Immunol. Methods* **1991**, *139*, 107–114. [CrossRef]
26. Mylonas, K.J.; Nair, M.G.; Prieto-Lafuente, L.; Paape, D.; Allen, J.E. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J. Immunol.* **2009**, *182*, 3084–3094. [CrossRef]
27. Manzanero, S. Generation of mouse bone marrow-derived macrophages. *Methods Mol. Biol.* **2012**, *844*, 177–181. [CrossRef]
28. Bogdan, C. Nitric oxide and the immune response. *Nat. Immunol.* **2001**, *2*, 907–916. [CrossRef]
29. Paiva, C.N.; Feijó, D.F.; Dutra, F.F.; Carneiro, V.C.; Freitas, G.B.; Alves, L.S.; Mesquita, J.; Fortes, G.B.; Figueiredo, R.T.; Souza, H.S. Oxidative stress fuels Trypanosoma cruzi infection in mice. *J. Clin. Investig.* **2012**, *122*, 2531–2542. [CrossRef]
30. Abrahamsohn, I.A.; Coffman, R.L. Trypanosoma cruzi: IL-10, TNF, IFN-γ, and IL-12 regulate innate and acquired immunity to infection. *Exp. Parasitol.* **1996**, *84*, 231–244. [CrossRef]
31. van Vliet, S.J.; Bay, S.; Vuist, I.M.; Kalay, H.; García-Vallejo, J.J.; Leclerc, C.; van Kooyk, Y. MGL signaling augments TLR2-mediated responses for enhanced IL-10 and TNF-α secretion. *J. Leukoc. Biol.* **2013**, *94*, 315–323. [CrossRef] [PubMed]
32. Kawai, T.; Akira, S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **2011**, *34*, 637–650. [CrossRef] [PubMed]
33. Koch, P.D.; Miller, H.R.; Yu, G.; Tallarico, J.A.; Sorger, P.K.; Wang, Y.; Feng, Y.; Thomas, J.R.; Ross, N.T.; Mitchison, T. A High Content Screen in Macrophages Identifies Small Molecule Modulators of STING-IRF3 and NFkB Signaling. *ACS Chem. Biol.* **2018**, *13*, 1066–1081. [CrossRef] [PubMed]
34. Dlugosz, E.; Basalaj, K.; Zawistowska-Deniziak, A. Cytokine production and signalling in human THP-1 macrophages is dependent on Toxocara canis glycans. *Parasitol. Res.* **2019**, *118*, 2925–2933. [CrossRef] [PubMed]
35. MacKenzie, S.; Fernandez-Troy, N.; Espel, E. Post-transcriptional regulation of TNF-alpha during in vitro differentiation of human monocytes/macrophages in primary culture. *J. Leukoc. Biol.* **2002**, *71*, 1026–1032. [PubMed]

36. Sayan, M.; Mossman, B.T. The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. *Part. Fibre Toxicol.* **2016**, *13*, 51. [CrossRef]
37. Lawlor, K.E.; Vince, J.E. Ambiguities in NLRP3 inflammasome regulation: Is there a role for mitochondria? *Biochim. Biophys. Acta* **2014**, *1840*, 1433–1440. [CrossRef]
38. Ng, W.C.; Liong, S.; Tate, M.D.; Irimura, T.; Denda-Nagai, K.; Brooks, A.G.; Londrigan, S.L.; Reading, P.C. The macrophage galactose-type lectin can function as an attachment and entry receptor for influenza virus. *J. Virol.* **2014**, *88*, 1659–1672. [CrossRef]
39. Ding, D.; Yao, Y.; Zhang, S.; Su, C.; Zhang, Y. C-type lectins facilitate tumor metastasis. *Oncol. Lett.* **2017**, *13*, 13–21. [CrossRef]
40. Singh, S.K.; Streng-Ouwehand, I.; Litjens, M.; Kalay, H.; Saeland, E.; van Kooyk, Y. Tumour-associated glycan modifications of antigen enhance MGL2 dependent uptake and MHC class I restricted CD8 T cell responses. *Int. J. Cancer* **2011**, *128*, 1371–1383. [CrossRef]
41. Napoletano, C.; Zizzari, I.G.; Rughetti, A.; Rahimi, H.; Irimura, T.; Clausen, H.; Wandall, H.H.; Belleudi, F.; Bellati, F.; Pierelli, L.; et al. Targeting of macrophage galactose-type C-type lectin (MGL) induces DC signaling and activation. *Eur. J. Immunol.* **2012**, *42*, 936–945. [CrossRef] [PubMed]
42. Freire, T.; Zhang, X.; Deriaud, E.; Ganneau, C.; Vichier-Guerre, S.; Azria, E.; Launay, O.; Lo-Man, R.; Bay, S.; Leclerc, C. Glycosidic Tn-based vaccines targeting dermal dendritic cells favor germinal center B-cell development and potent antibody response in the absence of adjuvant. *Blood* **2010**, *116*, 3526–3536. [CrossRef] [PubMed]
43. Torres-Gutierrez, E.; Perez-Cervera, Y.; Camoin, L.; Zenteno, E.; Aquino-Gil, M.O.; Lefebvre, T.; Cabrera-Bravo, M.; Reynoso-Ducoing, O.; Bucio-Torres, M.I.; Salazar-Schettino, P.M. Identification of O-Glcnacylated Proteins in Trypanosoma cruzi. *Front. Endocrinol.* **2019**, *10*, 199. [CrossRef] [PubMed]
44. Campos, M.A.; Closel, M.; Valente, E.P.; Cardoso, J.E.; Akira, S.; Alvarez-Leite, J.I.; Ropert, C.; Gazzinelli, R.T. Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88. *J. Immunol.* **2004**, *172*, 1711–1718. [CrossRef] [PubMed]
45. Koo, S.J.; Chowdhury, I.H.; Szczesny, B.; Wan, X.; Garg, N.J. Macrophages Promote Oxidative Metabolism To Drive Nitric Oxide Generation in Response to Trypanosoma cruzi. *Infect. Immun.* **2016**, *84*, 3527–3541. [CrossRef] [PubMed]
46. Giorgi, M.E.; de Lederkremer, R.M. Trans-sialidase and mucins of Trypanosoma cruzi: An important interplay for the parasite. *Carbohydr. Res.* **2011**, *346*, 1389–1393. [CrossRef] [PubMed]
47. Garg, N.; Postan, M.; Mensa-Wilmot, K.; Tarleton, R.L. Glycosylphosphatidylinositols are required for the development of Trypanosoma cruzi amastigotes. *Infect. Immun.* **1997**, *65*, 4055–4060.
48. Rodrigues, M.M.; Oliveira, A.C.; Bellio, M. The Immune Response to Trypanosoma cruzi: Role of Toll-Like Receptors and Perspectives for Vaccine Development. *J. Parasitol. Res.* **2012**, *2012*, 507874. [CrossRef]
49. Campos, M.A.; Almeida, I.C.; Takeuchi, O.; Akira, S.; Valente, E.P.; Procopio, D.O.; Travassos, L.R.; Smith, J.A.; Golenbock, D.T.; Gazzinelli, R.T. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. *J. Immunol.* **2001**, *167*, 416–423. [CrossRef]
50. Oliveira, A.-C.; Peixoto, J.R.; de Arruda, L.B.; Campos, M.A.; Gazzinelli, R.T.; Golenbock, D.T.; Akira, S.; Previato, J.O.; Mendonça-Previato, L.; Nobrega, A. Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi. *J. Immunol.* **2004**, *173*, 5688–5696. [CrossRef]
51. Drummond, R.A.; Gaffen, S.L.; Hise, A.G.; Brown, G.D. Innate Defense against Fungal Pathogens. *Cold Spring Harb. Perspect. Med.* **2014**, *5*, a019620. [CrossRef] [PubMed]
52. Tian, J.; Yang, G.; Chen, H.Y.; Hsu, D.K.; Tomilov, A.; Olson, K.A.; Dehnad, A.; Fish, S.R.; Cortopassi, G.; Zhao, B.; et al. Galectin-3 regulates inflammasome activation in cholestatic liver injury. *FASEB J.* **2016**, *30*, 4202–4213. [CrossRef] [PubMed]
53. Gonçalves, V.M.; Matteucci, K.C.; Buzzo, C.L.; Miollo, B.H.; Ferrante, D.; Torrecilhas, A.C.; Rodrigues, M.M.; Alvarez, J.M.; Bortoluci, K.R. NLRP3 controls Trypanosoma cruzi infection through a caspase-1-dependent IL-1R-independent NO production. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2469. [CrossRef] [PubMed]
54. Dey, N.; Sinha, M.; Gupta, S.; Gonzalez, M.N.; Fang, R.; Endsley, J.J.; Luxon, B.A.; Garg, N.J. Caspase-1/ASC inflammasome-mediated activation of IL-1β–ROS–NF-κB pathway for control of Trypanosoma cruzi replication and survival is dispensable in NLRP3$^{-/-}$macrophages. *PLoS ONE* **2014**, *9*, e111539. [CrossRef]

55. Liu, W.; Yin, Y.; Zhou, Z.; He, M.; Dai, Y. OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading to NLRP3 inflammasome activation. *Inflamm. Res.* **2014**, *63*, 33–43. [CrossRef]
56. Chen, Y.J.; Wang, S.F.; Weng, I.C.; Hong, M.H.; Lo, T.H.; Jan, J.T.; Hsu, L.C.; Chen, H.Y.; Liu, F.T. Galectin-3 Enhances Avian H5N1 Influenza A Virus-Induced Pulmonary Inflammation by Promoting NLRP3 Inflammasome Activation. *Am. J. Pathol.* **2018**, *188*, 1031–1042. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

APENDICE II: ARTÍCULOS RELACIONADOS CON LOS MECANISMOS DE ACTIVACIÓN DE LOS MACRÓFAGOS

Articulo 3: José L. Reyes, Danielle T. Vannan, Bertus Eksteen, Imelda Juárez Avelar, Tonathiu Rodríguez, Marisol Ibet González, and Alicia Vázquez Mendoza.2018. Innate and Adaptive Cell Populations Driving Inflammation in Dry Eye Disease. Review Article. Mediators of Inflammation. IF 3.5

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 2532314, 12 pages
https://doi.org/10.1155/2018/2532314

Review Article

Innate and Adaptive Cell Populations Driving Inflammation in Dry Eye Disease

José L. Reyes,[1] **Danielle T. Vannan**,[2,3] **Bertus Eksteen**,[3] **Imelda Juárez Avelar**,[4] **Tonathiu Rodríguez**,[4] **Marisol Ibet González**,[1] **and Alicia Vázquez Mendoza**[5]

[1]*Universidad Nacional Autónoma de México (UNAM), Laboratorio de Inmunología experimental y Regulación de la inflamación Hepato-Intestinal, UBIMED, Facultad de Estudios Superiores (FES) Iztacala, Tlalnepantla, MEX, Mexico*
[2]*University of Calgary, Snyder Institute for Chronic Diseases, Cumming School of Medicine, Calgary, AB, Canada*
[3]*Aspen Woods Clinic, Calgary, AB, Canada*
[4]*Universidad Nacional Autónoma de México (UNAM), Unidad de Biomedicina (UBIMED), Facultad de Estudios Superiores (FES) Iztacala, Tlalnepantla, MEX, Mexico*
[5]*Universidad Nacional Autónoma de México (UNAM), Laboratorio de Enfermedades Inflamatorias Oculares, Carrera de Optometría, Facultad de Estudios Superiores (FES) Iztacala, Tlalnepantla, MEX, Mexico*

Correspondence should be addressed to José L. Reyes; jlreyes@ired.unam.mx
and Alicia Vázquez Mendoza; aliciavm@ired.unam.mx

Academic Editor: Torben L. Sørensen

Copyright © 2018 José L. Reyes et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Dry eye disease (DED) is the most common ocular disease and affects millions of individuals worldwide. DED encompasses a heterogeneous group of diseases that can be generally divided into two forms including aqueous-deficient and evaporative DED. Evidence suggests that these conditions arise from either failure of lacrimal gland secretion or low tear film quality. In its secondary form, DED is often associated with autoimmune diseases such as Sjögren's syndrome and rheumatoid arthritis. Current treatment strategies for DED are limited to anti-inflammatory medications that target the immune system as the source of deleterious inflammation and tissue injury. However, there is a lack of understanding of the underlying pathogenesis of DED, and subsequently, there are very few effective treatment strategies. The gap in our knowledge of the etiology of primary DED is in part because the majority of research in DED focused on secondary autoimmune causes. This review focuses on what is currently understood about the contribution of innate and adaptive immune cell populations in the pathogenesis of DED and highlights the need to continue investigating the central role of immunity driving DED.

1. Introduction

1.1. Definition and Diagnosis of DED. Dry eye disease (DED) is a multifactorial condition involving the ocular surface, lacrimal glands, and meibomian glands leading to abnormal tear film quantity and/or quality. Characterized by discomfort and visual disturbance, DED may lead to loss of vision caused by exposure of the ocular surface to excessive desiccant stress. Due to the high prevalence of DED worldwide, it is a critical public health issue [1–3].

The Dry Eye Workshop (DEWS) has divided DED into two major classes: aqueous tear-deficient dry eye disease and evaporative dry eye disease. The former is often associated with autoimmune Sjögren's syndrome (ssDED) and is characterized by dysfunction of both lacrimal and salivary glands resulting in reduced tear secretion rate and/or volume. In evaporative DED, there is excessive fluid loss from the exposed ocular surface in the presence of normal lacrimal secretory function. The development of evaporative DED has also been associated with intrinsic factors such as meibomian oil deficiency, disorders of the eyelid, and low blink rate. Extrinsic factors that can also influence DED onset include vitamin A deficiency, topical drug preservatives, contact lens wearing, certain prescription drugs, and seasonal allergies [4].

Accurate diagnosis of DED requires completion of the ocular surface disease index (OSDI) questionnaire that addresses potential risk factors as well as clinical tests to assess tear production and elimination, tear stability, and ocular surface integrity [5, 6]. Advanced tools including confocal microscopy, vision function, and conjunctival cytology can also be applied to improve diagnostic accuracy of DED [7–9].

1.2. Dry Eye Disease Immunobiology. Studies have described certain immunomodulating factors involved in maintenance of the ocular surface that may be disrupted during DED. Recently, it was identified that retinoic acid, a metabolite of vitamin A, is critical for induction of $Foxp3^+$ T regulatory cells and contributes to the immune privilege of the eye [10]. Additionally, tear hyperosmolarity, hormonal changes, and mechanical irritation can also contribute to the onset of DED pathology [11–14]. Studies have shown that an imbalance of tear film components such as higher concentrations of sodium can result in increased osmolarity of the tear film and lead to inflammation with the potential to damage the ocular surface in part due to goblet cell apoptosis [15–17]. Persistent hyperosmolarity is further sustained by increased concentration of inflammatory cytokines and matrix metalloproteinases (MMPs) [18].

Therefore, inflammation associated with the eye has attracted interest from researchers worldwide in an effort to understand the immunological processes associated with the development of DED.

As in many chronic diseases, sustained or dysregulated inflammation consisting of increased proinflammatory cytokine levels and infiltration of immune cells has been identified. Therefore, targeting immune cells or inflammatory mediators may have therapeutic potential. Unfortunately, little is known about the specific pathogenic cell populations in DED.

In this review, unless stated otherwise, we are indicating DED as independent from autoimmune Sjögren's syndrome. We have summarized the available evidence for the role of both innate and adaptive cell populations as well as cytokines, chemokines, and their respective receptors in the pathology of DED.

2. Search Methodology

The following keywords were used to search the Pubmed database: dry eye disease, desiccant stress, ocular surface inflammation, and non-autoimmune. Papers published from 2000 to 2017 were reviewed. Limited focus was placed on Sjögren's syndrome-associated DED since there are already excellent reviews published [19, 20].

3. Innate Immunity Is a Driving Force in the Pathogenesis of DED

The innate inflammatory response is required to eliminate potential harmful pathogens and can contribute to tissue remodeling after injury. However, if dysregulated, this type of immune response may lead to sustained inflammation resulting in compromised host organ functions. Thus, understanding the inflammatory pathways activated during DED may provide insight to potential therapeutic targets.

3.1. Neutrophils. Neutrophils are short-lived polymorphonuclear cells (PMNs) predominantly associated with frontline resistance against pathogens. In addition to their ability to phagocytize potentially harmful antigens, neutrophils can activate potent antimicrobial defense mechanisms such as the release of reactive oxygen species (ROS) and extracellular neutrophilic DNA traps (NETs) [21–23].

Neutrophils have been detected on the ocular surface in patients with DED and are often colocalized with histone, neutrophil elastase, and extracellular DNA (eDNA) consistent with NET release. However, whether their presence is pathogenic or regulatory remains untested [24]. A recent study evaluating the presence of DNAse 1 in the lacrimal gland compared between a cohort of patients with symptomatic, tear-deficient DED, and asymptomatic individuals with normal tear production. The authors observed a significant decrease in the concentration of nucleases present in the tear fluid from patients with DED compared to healthy individuals. In addition, patients with DED had greater accumulation of eDNA on their ocular surface compared to the healthy controls. It is possible that the lack of nucleases and accumulation of eDNA and NETs on the precorneal surface may contribute to ocular surface inflammation in these patients (see Figure 1). This report is the first to suggest that by-products of neutrophil activation may contribute to DED in patients; however, further investigation is required to confirm these observations [24].

Previously, it has been established that tear hyperosmolarity can trigger DED; however, whether this alters neutrophil responses is not completely understood. Tibrewal et al. reported that hyperosmolar stress, generated by increasing concentration of NaCl, induced NETosis compared to cells incubated in isoosmolar media. Furthermore, neutrophils exposed to hyperosmolar media exhibited morphological changes such as larger and rounded nuclei occupying the majority of the cell. In order to confirm the impact of hyperosmolar conditions on NET formation, the authors restored isoosmolar conditions, and subsequently, NET formation decreased. Furthermore, the addition of NET formation inhibitors (i.e., staurosporine and anti-β2 integrin) resulted in lower numbers of NETs under hyperosmolar conditions. An association between hyperosmolarity and NET formation has been demonstrated and suggests that there may be a potential benefit of NET inhibition during DED [25].

Neutrophils are known for eliciting a robust immune response by responding to chemokines and inducing the release of proinflammatory mediators. In a mouse model of DED involving corneal damage by alkali burn, an increased production of inflammatory mediators such as interleukin (IL)-1β, IL-6, and matrix metalloproteinases (MMPs) and CXCL1 chemokine in whole corneal homogenates was observed. Analysis of myeloperoxidase (MPO) activity in corneal lysates suggested a significant increase in neutrophils (see Figure 2). When the mice were topically treated with either dexamethasone (Dex) or doxycycline (Doxy), there was a reduction in tissue injury and inflammatory mediators.

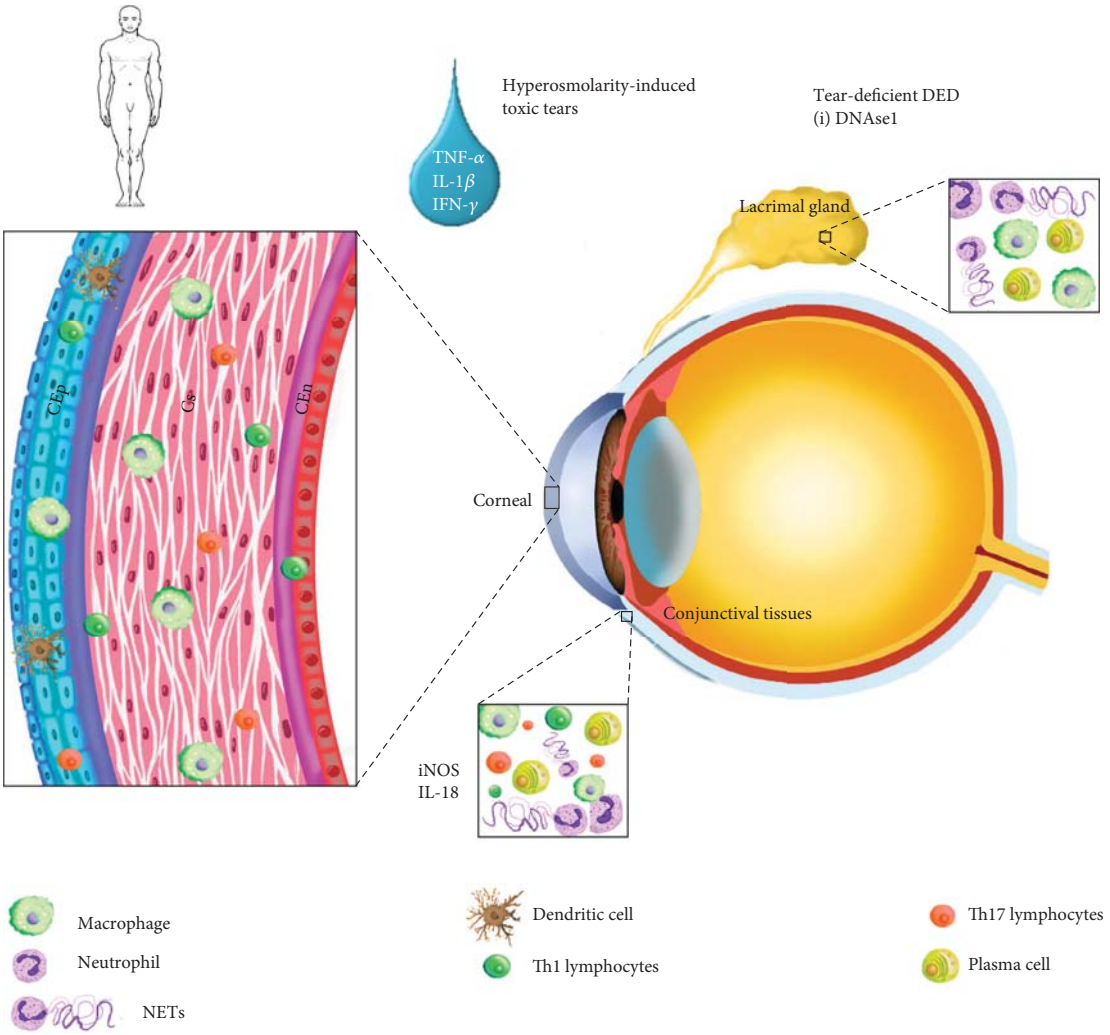


Figure 1: Schematic view of humoral and cellular inflammatory components found increased in patients with DED. Abbreviations: En: corneal endothelium; CEp: corneal epithelium; Cs: corneal stroma; IFN: interferon; iNOS: inducible nitric oxide synthase; NETs: neutrophil-derived extracellular traps; TNF: tumor necrosis factor.

While both treatments attenuated the degree of inflammation, a differential effect was observed, that is, Dex treatment significantly decreased IL-6; MMPs −1, −3, −9, and −13; and TIMP-1 but enhanced MMP-8 transcripts up to 1000-fold. In contrast, Doxy treatment showed greater suppression of IL-6, MMP-8, MMP-9, and MMP-13. Moreover, Dex-treated corneas had a significant decrease in $Gr1^+$ cell counts as compared to vehicle-treated corneas at days two and five postinjury. Therefore, corneal opacity was associated with neutrophil infiltration, most likely, CXCL-1 mediated [26].

Sex-associated changes in neutrophils influencing the susceptibility to DED have also been identified in mouse models. Inhibiting lacrimal gland function using scopolamine in female mice resulted in more severe DED and lower numbers of lipoxin A4- (LXA4-) producing PMNs in the corneal limbus, lacrimal gland, and cervical lymph nodes compared to male mice. This also correlated with heightened expansion of IFN-γ- and IL-17-producing $CD4^+$ T cells and a significant decrease in $Foxp3^+$ regulatory T cells (see Figure 2). Additionally, when neutrophils were depleted by intraperitoneally delivered anti-Ly6G antibodies, levels of LXA4 decreased concomitantly with uncontrolled proliferation of Th1 and Th17 $CD4^+$ cells. Interestingly, topical and systemic administration of LXA4 restored numbers of $Foxp3^+$ regulatory T cells and drastically suppressed Th1 cells and to a lesser degree Th17 cells resulting in attenuated DED. Thus, a previously unknown sex-specific, T regulatory-promoting function via LXA4 release in neutrophils was uncovered [27].

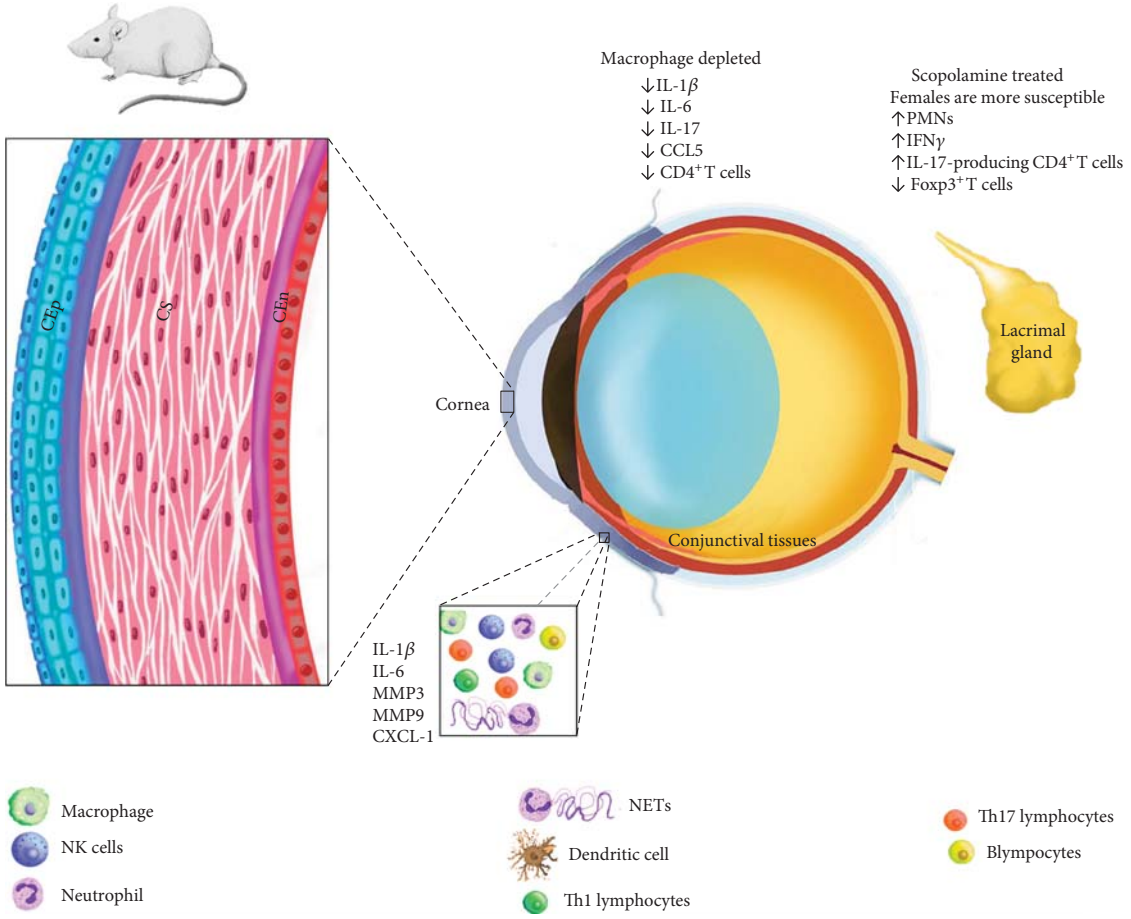


Figure 2: Schematic view of humoral and cellular inflammatory components found in experimental DED. Abbreviations: CEn: corneal endothelium; CEp: corneal epithelium; Cs: corneal stroma; IFN: interferon; iNOS: inducible nitricoxide synthase; MMP: matrix metalloprotease; NETs: neutrophil-derived extracellular traps; PMN: plymorphonuclear; TNF: tumor necrosis factor.

Sjögren's syndrome is an autoimmune-mediated eye pathology where one of the main identified autoantigens is the 48KDa ribonucleoprotein, also known as antigen B. Interestingly, antigen B is expressed in the nucleus and surface membrane of human neutrophils which is released when neutrophils undergo apoptosis. Whether targeting neutrophils worsens disease outcome has not been explored; however, it has been shown that antigen B activates the MAP kinase pathway and induces IL-8 output from a donor neutrophil and a neutrophil cell line [28].

Growing evidence demonstrates that neutrophil infiltration occurs in patients with DED as well as in experimental murine models with potentially deleterious outcomes. To date, a role for early infiltrating neutrophils has been shown. However, further research is required to clarify if early versus late cellular recruitment of neutrophils can drive differential function within the tissue. Understanding more about the neutrophil phenotypes similar to other innate immune cells like macrophages is required to direct future development of therapeutic targets.

3.2. Macrophages. Macrophages are innate immune cells with a wide variety of functions ranging from recognition and clearance of pathogens to activation and polarization of adaptive immune cells. Other macrophage features include tissue repair and secretion of growth factors promoting angiogenesis [29]. Macrophages are highly plastic cells as evidenced by differential responses exerted by these cells depending on encountering either Th1- (IFN-γ-) or Th2- (IL-4-) dominated environments. Macrophages contribute to the spectrum of immune responses by acquiring polarized phenotypes that can be broadly classified as classically activated (M1) or alternatively activated (M2) macrophages [30, 31]. The role of macrophages has recently been addressed in DED particularly associated with immune-mediated eye injury.

In Sjögren's syndrome, both lacrimal and salivary glands can become severely damaged. Manoussakis et al. found that when comparing patients with DED, macrophage infiltration in the periepithelial area was greater in samples from patients with Sjögren's syndrome than patients without. In contrast, dendritic cell (DC) distribution was mostly intraepithelial with consistently higher numbers in patients with Sjögren's syndrome. Interestingly, periepithelial $CD68^+$ macrophages were found to also be positive for IL-18, which positively correlated with glandular inflammation [32]. In a separate study, minor salivary gland (MSG) biopsies from patients with primary Sjögren's syndrome (pSS) had increased numbers of $CD68^+$ macrophages that correlated with the grade of inflammation and neovascularization [33]. Therefore, in the case of autoimmune Sjögren's syndrome, there appears to be a role for macrophages to infiltrate the ocular tissue and contribute to eye inflammation.

Due to the plastic nature of macrophages, You et al. evaluated the presence and polarity of macrophages during desiccant stress-induced DED in the cornea and conjunctival epithelium. The authors did not detect any significant changes in the number of macrophages over time in either the cornea or conjunctiva. Measurement of canonical markers of macrophage activation in conjunctival tissue demonstrated that M1-associated marker iNOS expression was increased and paralleled IL-18 high levels on day 10 post-injury; however, arginase-1 remained unchanged. This data suggests that there may be a time-dependent effect on macrophage phenotypes in DED [34]. In line with this, others have also reported M1-like cells in the salivary gland during experimental Sjögren's syndrome [35]. However, care should be taken in interpreting differences in macrophages during DED as Zhou et al. have shown increased macrophage infiltration in the corneal limbus in AIRE-deficient mice, a systemic autoimmune model affecting the lacrimal glands. They reported substantial infiltration of macrophages into the cornea, and when depleted with locally delivered clodronate-loaded liposomes, corneal tissue thickness and opacity were attenuated. Others have also reported that macrophage depletion attenuates experimental models of DED predominantly by decreasing inflammatory mediators (e.g., IL-1β, IL-6, IL-17, and CCL5) and $CD4^+$ T cells [36]. Taken together, this data suggests that macrophages likely contribute to inflammation present in autoimmune-mediated DED whereas the data is not as consistent for nonautoimmune cases of DED [37].

Changes in circulating monocytes have also been observed during DED. In a report by Hauk et al., they showed that $CD14^+$ monocytes isolated from peripheral blood in patients with pSS had reduced ability to phagocytize apoptotic salivary gland epithelial cells compared to monocytes from healthy donors. The monocytes exhibited heightened expression of vasoactive intestinal peptide (VIP) receptor 2 (VPAC2) but not VPAC1. However, when testing monocyte response to VIP, neither phagocytic ability nor inflammatory mediator release (TNF-α) was altered in the presence of recombinant VIP [38]. Authors highlight the fact that VPAC2 overexpression in monocytes might reflect compromised monocyte features upon apoptotic cell clearance (i.e., aberrant secretion of TNF-α rather than TGFβ release).

3.3. NK Cells. Natural killer (NK) cells are a group of innate lymphocytes with cytotoxic function including secretion of membrane-disrupting granzyme and perforin largely aimed at clearing transformed and infected host cells [39–41]. Furthermore, they have proven to influence the immune response by secreting large amounts of cytokines, mainly IFN-γ, resulting in activation of neighboring cell populations such as macrophages and T cells. NK cells have been widely studied under a variety of pathologies ranging from infectious to autoimmune [42–45]. Although the role of NK cells has been explored in Sjögren's syndrome, little is known in regard to their role in DED outside of an autoimmune disease context. A cohort of 106 patients with DED was evaluated to determine the expression of inhibitory receptors for NK cells known as KIRs. It was found that the inhibitory NK cell receptor KIR2DS2 in combination with HLA-C1 allele, but not other KIRs, was frequently associated with patients suffering from severe DED compared to healthy donors suggesting an underlying genetic link between NK cells and DED susceptibility in patients [46]. In a pioneering study, Chen et al. reported an early increase in NK1.1 transcript expression in conjunctival samples from chemically induced DED in mice that was later confirmed by flow cytometry ruling out the contribution from NKT cells ($NK1.1^+$ $TCR\beta^-$). The percentage of conjunctival NK cells was three-fold higher in mice with DED than control mice (see Figure 2). In contrast, there was no difference in the number of NK cells present in draining lymph nodes between mice with DED and control mice; however, the number of IFN-γ-producing NK cells isolated from the lymph nodes was higher in DED mice as compared to control mice. Authors also found that neither NK cell depletion nor IFN-γ immunoneutralization correlated with lower corneal surface injury and decreased levels of TNF-α and IFN-γ cytokines in mice with DED. Interestingly, mice lacking NK cells exhibited lower levels of costimulatory molecules (CD80, CD86, and MHCII) on APCs. In the context of nonautoimmune DED, NK cell conjunctival infiltration and disease promotion are possibly driven by IFN-γ secretion and ultimately impact APC maturation and disease outcome [47].

A further role for NK cells contributing to DED was described by Zhang et al. Intraepithelial NK cells were found to reside in the conjunctival tissue and are further expanded upon desiccant stress caused by scopolamine administration and airflow exposure (see Figure 2). Subsequent depletion of NK cells results in lower numbers of IL-17A-producing $CD4^+$ T cells in the ocular surface and cervical lymph nodes five days after desiccant stress exposure. Furthermore, in the absence of NK cells, authors noticed reduced expansion of $CD11b^+MHCII^+$ and $CD11c^+MHCII^+$ antigen-presenting cells (APCs). In order to confirm a central role for NK cells in Th17 expansion, Th17 cells from the spleen and cervical lymph nodes were harvested and transferred into nude mice depleted of NK cells, which later were protected against disruption of the corneal barrier. In contrast, the transfer of Th17 cells in the presence of intact NK cells developed ocular

surface injury in mice. This report highlights the important role for NK cells in modulating APC activity leading to polarization of pathogenic Th17 cells involved in ocular surface inflammation [48].

4. Adaptive Immunity in DED

4.1. Conventional $CD4^+$ T Lymphocytes. T cells and antibody-producing B cells are the main constituent cells of the adaptive immune system. T lymphocytes can be further divided in numerous subsets including the two best characterized subpopulations $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells. As such, these cells are considered drivers and effectors in ongoing immune responses. Although their role is central in resistance to pathogens, both $CD4^+$ and $CD8^+$ T cells have been implicated in numerous autoimmune and autoinflammatory diseases. A critical role for subsets of $CD4^+$ T cells has been described in DED as well as Sjögren's syndrome.

The recruitment of T lymphocytes into inflamed conjunctival tissue from patients with moderate to severe DED has been characterized. Although the number of infiltrating $CD4^+$ and $CD8^+$ T cells was similar between patients with as Sjögren's syndrome DED and nonautoimmune DED, markers of T cell recruitment and activation were upregulated only in patients with nonautoimmune DED. In addition, ICAM expression was upregulated in conjunctival epithelial cells. This data suggests an important role for $CD4^+$ T cells and integrins contributing to peripheral T cell migration and proliferation in patients with DED [15].

Ethnicity may also play a role in the differences between T lymphocytes in autoimmune and nonautoimmune DED. In a cohort of patients from Korea, authors reported a more severe phenotype of Sjögren's syndrome DED patients as compared to nonautoimmune DED patients [49]. In addition, the patients had increased CXCL11 protein levels in tear samples and $CD4^+CXCR3^+$ Th1 lymphocytes assayed by flow cytometry in conjunctival tissue. Thus, despite of differences in the autoimmune or nonautoimmune origin of DED, reports consistently demonstrate abundant infiltrating $CD4^+$ T cells in ocular tissue suggesting that T cell infiltration might be a requisite to maintain the inflammatory process observed in DED.

An extensive analysis of T cells in conjunctiva samples from healthy individuals and patients with DED in Singapore was recently conducted. Bose et al. characterized T cells from healthy donors by collecting impression cytology samples (noninvasive biopsy of the conjunctival surface) and identified a clear $CD8^+$ T cell dominance over $CD4^+$ T cells. In addition, healthy conjunctivae were found to contain a resident population of $CD8^+/CD69^+/CD103^+/CCR7^-$ T lymphocytes. Using an array of T cell-specific surface markers, authors further characterized different T cell subsets present on the ocular surface from 52 patients with DED. Using these markers, patients were able to be clustered into different categories. Cluster 1 DED patients had high ocular redness and increased conjunctival $CD8^+$ ($T_{\text{Central Memory}}$) cells whereas cluster 2 DED patients had increased tear instability and higher proportions on conjunctival $CD4^+$ ($T_{\text{Effector Memory}}$) cells. Interestingly, most of the patients were grouped in cluster 2; however, when grouped based on DED subtype (i.e., aqueous deficient, evaporative, or mixed), 21% of patients did not fall into either cluster due to a unique DED phenotype with unaltered canonical DED tests (Schirmer's and break up time test) [50]. Thus, it was shown that the complex nature of DED may lead to a redistribution of T cell populations compared to T cells from samples of healthy ocular tissue.

Murine models of DED including the scopolamine-induced desiccant stress model have been employed to study the relationship between T lymphocytes and ocular inflammation. Niederkorn et al. reported that the transfer of $CD4^+$ T cells from the spleen and lymph nodes of sensitized mice into nude mice resulted in a strong ocular-specific inflammatory response characterized by inflammatory foci in the cornea, conjunctiva, and lacrimal gland along with a significant decrease in the number of goblet cells and tear volume. Transfer of $CD4^+$ T cells elicited inflammation on both the ocular surface and lacrimal gland even in the absence of airflow and desiccant stress. This study demonstrated that ocular surface-specific T cells are rapidly generated upon scopolamine sensitization and have the ability to induce DED in mice lacking T cells, suggesting the emergence of autoreactive $CD4^+$ T cells. In addition, authors found that cotransfer of $CD25^+$ T regulatory cells lead to a protective effect whereas this protection was lost upon depletion of Tregs during the transfer. The protective effect of T regulatory cells was also associated with a reduction in neutrophil infiltration [51].

4.2. $CD4^+$ Th17 Lymphocytes. A tremendous focus has been placed on the Th17 response in numerous inflammatory diseases [52–54]. In the case of DED, De Paiva et al. observed a strong expression of the IL-23/Th17 axis including the Th17-promoting cytokines IL-6, IL-23R, TGFβ2, and RORγt transcription factor (expressed by committed Th17 cells) in DED patients. In confirmation, using the scopolamine-induced DED mouse model, authors found increased levels of IL-17 in tear samples from mice with DED and abundant ocular surface IL-17-producing cells. Neutralization of IL-17 led to an attenuation of DED in mice; however, the cellular source of IL-17 was not conclusively determined [55].

More recently, the presence of Th17 cells and their role in driving DED were addressed by means of chemically induced experimental DED. Dohlman et al. demonstrated significant expansion of IL-17-secreting $CD4^+$ T cells rather than Th1 (IFN-γ-secreting T cells) in draining lymph nodes from animals receiving desiccant stress for 12 days. In addition, authors confirmed the presence of CCR6 chemokine receptor on the surface membrane of IL-17-secreting $CD4^+$ T cells. The authors further quantified the expression of the CCR6 ligand CCL20 on corneal and conjunctiva tissue. Functional assays aimed to test the importance of the CCR6-CCL20 axis in DED were conducted by immune neutralization of subconjunctival-delivered blocking anti-CCL20 antibody which resulted in reduced numbers of Th17 cells in both draining lymph nodes and conjunctival samples. Moreover, this later correlated with improved clinical signs in DED mice (reduced corneal epitheliopathy) and decreased mRNA expression of inflammatory mediators such as IL-6, MMP3,

and IFN-γ in corneal and conjunctival tissues [56]. The impact of Th1- and Th17-specific chemokine receptors, CXCR3 and CCR6, respectively, in DED outcome was also measured. The authors found a high percent of $CCR6^+CD4^+$ T cells in both cervical lymph nodes and the ocular surface five days after desiccant stress induction whereas $CXCR3^{+-}$ $CD4^+$ T cells are seen increased only on the ocular surface. The role of these CCR6 and CXCR3 chemokine receptors was tested in mice lacking either receptor. Unlike wild-type mice that exhibited compromised corneal integrity associated with increased conjunctiva infiltrating $CD4^+$ cells and reduced goblet cell hyperplasia, mice lacking either CCR6 or CXCR3 were found to have preserved corneal integrity alongside a suppressed $CD4^+$ cell response and unaltered goblet cell density. Additionally, IL-17 and IFN-γ responses were high in cervical lymph nodes but suppressed in the ocular surface in the absence of both chemokine receptors (CCR6 and CXCR3). To test the reduced pathogenicity attributed to T cells lacking either of the chemokine receptors, adoptive transfer experiments were carried out in T cell-deficient RAG-1 mice. Lymphocytes harvested from DED mice were transferred into naïve RAG-1 recipient animals and disease assessed. Transfer of chemokine receptor-deficient T cells inhibited the infiltration of $CD4^+$ T cells into the conjunctival epithelium and preserved goblet cell density. When inflammatory cytokine expression was assayed, authors found that mice transferred with chemokine receptor-deficient T cells displayed a clear inhibition of virtually all the cytokines (IL-6, IL-13, IL-17, and IFN-γ) and MMP 3 and 9 tested in corneal and conjunctival tissue [57].

Recently, it was identified that GM-CSF released by Th17 cells promotes recruitment and activation of $CD11b^+$ myeloid cells in experimental DED. Analysis of cornea and conjunctiva tissues showed heightened mRNA expression and protein levels of GM-CSF upon DED induction. Intracellular flow cytometry confirmed double positive staining for IL-17 and GM-CSF. To test the putative role of GM-CSF on DED pathogenesis, $CD11b^+$ myeloid naive cells were exposed to supernatants harvested from anti-CD3-stimulated purified $CD4^+$ T cells from DED mice with or without anti-GM-CSF antibody. Only $CD11b^+$ cells incubated with supernatants in the presence of isotype control antibody displayed significant high levels of MHCII and Ki67 antigens indicating activation and proliferation, respectively, as compared to those cells incubated with supernatants in the presence of anti-GM-CSF-neutralizing antibodies. When anti-GM-CSF treatment was given locally to assess the *in vivo* impact on myeloid cells during DED development, authors reported that the treatment caused diminished infiltration of $CD11b^+MHCII^{hi}$ cells into the ocular surface and low numbers of Th17 cells in draining lymph nodes paralleled with attenuated clinical signs as gauged by improved corneal integrity [58]. Also, IL-17 itself has been proven to be an important growth factor for B cells in the context of DED as recently described by Subbarayal et al. This study provided evidence that Th17 cells obtained from DED animal cervical lymph nodes had the ability to induce B cell proliferation and antibody switch in a contact-independent IL-17-dependent manner. Therefore, the pathogenic mechanisms displayed by Th17 may impact the humoral response by targeting B cells [59]. Evidence generated from different groups points out to a highly pathogenic role of infiltrating Th17 cells via its interaction with both innate and adaptive cells.

5. B Lymphocytes

B cells also contribute to the adaptive immune response. These cells are pivotal given their ability to secrete cytokines and as APCs; however, their key role as antibody-producing cells places them as central players in immune surveillance as well as in response to pathogen invasion. For ocular surface homeostasis, it has been reported that plasma cells are abundant in the human diffuse conjunctiva-associated lymphoid tissue (CALT) and the lacrimal drainage-associated lymphoid tissue (LDALT) and in the lacrimal glands [60]. Plasma cells residing in the ocular surface continuously release secretory IgA which is one of the most important humoral components in mucosal protection. IgA is thought to be responsible for limiting ocular microbiota invasion into deeper tissues [60]. The presence of B cells in human samples under sterile conditions is well documented; however, in contrast, mouse studies reported B cells ($B220^+CD3^-$) as one of the least prevalent cell populations in the ocular surface [48].

B cells have been studied in eye diseases from autoimmune origins including Sjögren's syndrome [61] and uveitis [62]. B cell depletion therapy using anti-CD20 antibodies has also been used as a treatment in eye diseases [63] suggesting an important role for B cells in the immunopathogenesis of several autoimmune diseases in the eye. However, the exact role of B cells in terms of either experimental or clinical DED is unclear. Although it has been reported that plasma cells reside in the human eye-associated lymphoid tissue (EALT) [60], one study of patients with DED found no change in the number of B cells; rather, it showed changes in CD4 and CD8 T cells and epithelial cell activation [64].

In terms of experimental DED, several reports have attempted to dissect the role of B cells. Stern et al. described that anti-kallikrein 13 antibodies arise upon desiccant stress-induced DED [65]. Also, when whole serum or purified IgG from DED mice were transferred into mice lacking T cells, this resulted in complement-dependent DED in recipient mice, suggesting activation of autoreactive B cells in donor mice [65]. To date, whether a late autoimmune response is triggered in human DED is unknown. Also, it was determined that mice naturally developing DED are a consequence of aging reflected by increased numbers of B cells in the lacrimal gland and severe ocular alterations [66]. Interestingly, it has also been found that by using the same DED mouse model (i.e., pharmacological inhibition of the lacrimal gland), no changes in B cell population were observed at 5 and 10 days upon DED induction while other intraepithelial cells like T cells and NK cells are more represented in this microenvironment [66].

More recently, Subbarayal et al. showed that IL-17, which has been described as a highly pathogenic cytokine in DED, can also target B cells leading to their proliferation. Furthermore, neutralizing IL-17 reduced germinal center formation and the pathogenicity of transferred B cells [59]. Therefore,

it was shown that B cells can actively participate in promoting experimental DED.

Although B cells reside in the EALT, there is no clear consensus in regard to their specific participation in the immunopathology of DED. One can speculate that discrepancies between human and mouse DED mostly rely on the stage of DED, given that most of the mouse studies have addressed this in the context of acute DED (no more than 10 days post-scopolamine administration) whereas human studies are conducted in individuals being affected for longer periods of time by DED.

6. The Unexplored Role of EALT-Resident Granulocytes in DED

Due to the contact between mucosal surfaces and the environment, certain immune cells are strategically positioned to rapidly respond to pathogenic threats. Early study of the ocular surface in rodents found that mast cells are present on the ocular surface and that their presence early in prenatal development may possibly contribute to the normal morphogenesis of corneal tissue [67, 68]. However, mast cells may also have a pathogenic role in allergic disorders, uveitis, and viral infections [69–71]. Although it has been shown that mast cells may play a dual role (i.e., contributing in corneal morphogenesis and promoting eye type 2 inflammatory reactions), no data yet exists for the possible role of mast cell populations on DED development.

Pioneering studies on resident leukocyte populations in the conjunctiva demonstrated that eosinophils are absent in the healthy eye, but during an allergic reaction, eosinophils can infiltrate the ocular surface and contribute to a Th2 inflammatory response [72]. Eosinophils have been widely studied in allergic ocular reactions including giant papillary conjunctivitis and vernal conjunctivitis where it was observed that these cells contribute to the inflammatory responses by releasing histamine and cationic proteins. In addition, corneal and conjunctival fibroblasts, endothelial cells, and mast cells have the ability to express eosinophil-attractant chemokines like eotaxin 1 [69, 73]. In the absence of basophils, there can be impaired infiltration of eosinophils into the ocular surface suggesting that basophils also promote eosinophil trafficking into the eye [74].

Although basophils have been poorly studied in the settings of inflammatory ocular disorders, recently, Sugita et al. reported an important role played by early-produced type 2 cytokines (i.e., IL-33 and thymic stromal lymphopoietin (TSLP)) but not IL-25 in promoting calpain-induced eye inflammation. The authors showed that ocular damage is attenuated in the absence of both IL-33 and TSLP, such phenomenon correlated with a low number of infiltrating basophils whose depletion also diminished eosinophil infiltration [74]. Thus, although little is known about the role of basophils, these findings put basophils as an additional disease-promoting innate cell population, in part, via modulating eosinophil responses.

Strikingly, the relevant role of innate cells like the above-mentioned granulocytes in other inflammatory ocular diseases suggests that these cells are, most likely, also involved in generating tissue injury in response to desiccating stress, however, evidence still awaits to be shown.

7. Common Inflammatory Mediators in DED

Immune cell trafficking and activation employ soluble mediators that enable cells to infiltrate sites of tissue injury and modulate the immune response through receptor-ligand interactions. As immune system messengers, cytokines are key players in the stages of the inflammatory response. Examination of samples from patients with DED has revealed a pattern of inflammatory mediators associated with disease. Research groups have also identified a set of common mediators that can be detected in animal models of DED. A summary of these shared inflammatory mediators are shown in Table 1. Research to date demonstrates that Th1 and Th17 lymphocyte-associated mediators are predominant drivers in DED.

8. Future Directions

The eye contains numerous immune cells strategically positioned at the ocular surface with the primary goal of keeping the microenvironment tolerogenic to prevent unwanted tissue injury. In addition, resident immune cells also contribute to preserving the function of highly specialized cells involved in transduction of light stimuli into brain cells and conversion into images accomplishing the central function of the eye. Recently, reports have underscored the complex pattern of resident innate and adaptive immune cells interacting with ocular surface cells such as corneal and conjunctival epithelial cells, goblet cells, and stromal cells to maintain tear film integrity and corneal transparency which ultimately promotes ocular homeostasis. When ocular homeostasis breaks down, there is potential for the tolerogenic programs in resident cells to be altered and result in inflammatory processes that compromises both the tear film and ocular surface integrity. The triggers and mechanisms by which ocular tolerance disappears are starting to be understood, specifically in the context of DED.

The challenge to come is to better define the factors behind the onset of DED. Environmental factors such as exposure to temperature controlled air, low humidity, contact lens use, extended exposure to LED-based technologies, and recently diet and excessive antibiotic use are all being considered. Growth in our knowledge of the microbiome may also become a prominent area of study. Conjunctiva-resident bacteria have been linked to modulating DED, and efforts have been made to profile conjunctiva-resident microbiota under homeostatic conditions [75]. Starting to emerge is its potential as a biologic agent inhibiting pathogen proliferation due to colonization resistance [76]. Knowledge of bacterial communities promoting tolerance on ocular surfaces provides a potential new therapeutic alternative based on the success so far treating recurrent *C. difficile* infections and obesity [77]. Uncovering potential dysbiosis in ocular microbiota and the contribution to the pathogenesis of DED is required before implementation of novel therapies.

Table 1: Cytokines correlated with ocular inflammatory DED.

Host	Infiltrated immune cells	Inflammatory mediators on the ocular surface	References
Human (15 NS-KCS patients)	$CD4^+$ and $CD8^+$	⬆ ICAM-1	17
Human (5 moderate DED patients)	CEC	⬆ nondifferences (IL-1β, IL-8, IL-6, and ICAM)	69
Human (30 dysfunctional tear syndrome patients)	ND	⬆ IL-6, IL-8, and TNF-α	3
Human (23 evaporative-type DED patients (46 eyes))	ND	⬆ IL-1Ra, IL-6, and CXCL/IL-8	70
Human (17 DED patients)	$CD4^+$	⬆ CXCL9, 10, 11, and CXCR3	48
Human (35 aqueous-deficient dry eye DRY-aq patients, 36 lipid layer-deficient DRYlip patients, and 34 in combination of both (DRYaplip))	ND	⬆ IL-1β, IL-6, IL-8, TNF-α, and IFN-γ	71
Human (DED patients)	ND	⬆ IL-1β, IL-6, INF-γ, and TNF-α	72
Human (70 patients)	ND	⬆ IFN-γ	73
Human (34 DED patients with HIV infection and 32 DED patients without HIV infection)	ND	⬆ EGF, IFN-gamma-inducible protein 10 (IP-10, CXCL10)	74
Human (19 diabetic patients with DED and 15 nondiabetic patients with DED)	ND	⬆ IL-β and TNF-α	75
Human (15 samples in non-SS patients)	ND	⬆ IL-6, IL-17-A, and IL-23	76
Human (DED)	$CD4^+$ and $CD8^+{}_{RM}$	⬆ $CCR7^-$ T_{EM}	49
Human (32 patients severe DED)	ND	⬆ IL-17 and TNF-α	77
Murine (BALB/c, T cell-deficient nude BALB/c (BALB/cByJHfH11<υ>), and C57BL/6)	Neutrophil, mononuclear cell, and $CD4^+$	ND	22
Murine (C57Bl/6)	$CD4^+$	⬆ IL-6, IL-17 mRNA	78
Murine (C57BL/6 desiccating stress)	$CD4^+IL\text{-}17^+$	⬆ TNF-α, IL-1β, IL-6, IFN-γ, IL-23, and IL-17A	53
Murine (C57BL/6)	$CD11b^+$, $CD4^+$IL-IFN-γ^+, and $CD4^+IL\text{-}17^+$	⬆ CCR6, CCL20, and IL-17A	54
Murine	$CD11b^+$ and $CD4^+IL\text{-}17^+$	⬆ IL-17A and GM-CSF	56
Murine (C57BL/6)	$CD4^+CXCR3^+$ T cells	⬆ IL-1β, Il-6, TNF-α, IFN-γ, and ROS	79

DED: dry eye disease; ND: nondetermined; Mo: macrophages; non-SS: non-Sjögren syndrome; EGF: epidermal growth factor and IFN-gamma-inducible protein 10 (IP-10, CXCL10); CEC: conjunctival epithelial cells; NS-KCS: non-Sjögren's syndrome keratoconjunctivitis sicca; T_{RM}: resident memory T cells, T_{EM}: effector memory T cells; ROS: reactive oxygen species.

Future treatment strategies may also be developed based on existing therapeutic options for autoinflammatory diseases such as inflammatory bowel disease (IBD). In the case of IBD, application of cytokine-targeting therapies including anti-TNF-α therapy may have the potential to reduce DED clinical signs. Thus, identifying key cytokines driving ocular inflammation may guide the development of novel cytokine-based targeted therapies. Specifically, we recently found that the absence of macrophage migration inhibitory factor (MIF) protects against decreased tear volume and preserves tear's mucin patterns and goblet cell numbers in a scopolamine-induced DED mouse model (unpublished data). In addition, targeting nonimmune cells like goblet cells that are central in keeping tolerance through TGFβ release and mucin production opens the possibility of exploiting goblet cell expansion as another way to attenuate ocular surface inflammation.

9. Concluding Remarks

We presented numerous studies describing the complex manner for how the eye is activated upon desiccating environmental stimuli and it also has the ability to further amplify such aggressive reactions observed in immunopathogenesis of experimental and human DED. Currently, anti-inflammatory drugs and artificial tears are the most often used treatment for DED demonstrating the importance of immune response during this disease. Unfortunately, the immunological landscape is not completely understood which limits the development of new therapeutic agents. Further, DED itself is a multifactorial idiopathic disease which makes it even more complicated to treat. New therapeutics directed at counteracting DED symptoms and deleterious effects caused by sustained inflammation on ocular surface are required.

In summary, inflammatory cellular infiltrate is a prominent characteristic of DED and it has been described that both innate and adaptive cells transmigrate and trigger DED. Continued examination is needed to reveal novel targets for development of effective therapeutics.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

José L. Reyes is currently financially supported by Programa de Apoyo a los Profesores de Carrera (PAPCA) FESI-DIP-PAPCA-2016-24 and Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT) proyecto IA204618. Alicia Vázquez Mendoza is funded by PAPIIT TA200118.

References

[1] Q. Le, X. Zhou, L. Ge, L. Wu, J. Hong, and J. Xu, "Impact of dry eye syndrome on vision-related quality of life in a non-clinic-based general population," *BMC Ophthalmology*, vol. 12, no. 1, p. 22, 2012.

[2] B. Miljanovic, R. Dana, D. A. Sullivan, and D. A. Schaumberg, "Impact of dry eye syndrome on vision-related quality of life," *American Journal of Ophthalmology*, vol. 143, no. 3, pp. 409–415.e2, 2007.

[3] H. Lam, L. Bleiden, C. S. de Paiva, W. Farley, M. E. Stern, and S. C. Pflugfelder, "Tear cytokine profiles in dysfunctional tear syndrome," *American Journal of Ophthalmology*, vol. 147, no. 2, pp. 198–205.e1, 2009.

[4] "The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007)," *The Ocular Surface*, vol. 5, no. 2, pp. 75–92, 2007.

[5] M. J. Kim, S. S. Stinnett, and P. K. Gupta, "Effect of thermal pulsation treatment on tear film parameters in dry eye disease patients," *Clinical Ophthalmology*, vol. 11, pp. 883–886, 2017.

[6] V. Satitpitakul, A. Kheirkhah, A. Crnej, P. Hamrah, and R. Dana, "Determinants of ocular pain severity in patients with dry eye disease," *American Journal of Ophthalmology*, vol. 179, pp. 198–204, 2017.

[7] C. L. Mocanu, S. Jurja, A. G. Deca et al., "Impression conjunctival cytology in sicca syndrome - correlations between clinical and histological findings related to dry eye severity," *Romanian Journal of Morphology and Embryology*, vol. 57, no. 1, pp. 197–203, 2016.

[8] J. Hong, W. Zhu, H. Zhuang et al., "In vivo confocal microscopy of conjunctival goblet cells in patients with Sjogren's syndrome dry eye," *The British Journal of Ophthalmology*, vol. 94, no. 11, pp. 1454–1458, 2010.

[9] T. Kojima, Y. Matsumoto, M. Dogru, and K. Tsubota, "The application of in vivo laser scanning confocal microscopy as a tool of conjunctival in vivo cytology in the diagnosis of dry eye ocular surface disease," *Molecular Vision*, vol. 16, pp. 2457–2464, 2010.

[10] R. Zhou, R. Horai, P. B. Silver et al., "The living eye "disarms" uncommitted autoreactive T cells by converting them to $Foxp3^+$ regulatory cells following local antigen recognition," *Journal of Immunology*, vol. 188, no. 4, pp. 1742–1750, 2012.

[11] X. Jin, Z. Lin, Y. Liu, L. Lin, and B. Zhu, "Hormone replacement therapy benefits meibomian gland dysfunction in perimenopausal women," *Medicine*, vol. 95, no. 31, article e4268, 2016.

[12] M. Rolando, M. F. Refojo, and K. R. Kenyon, "Increased tear evaporation in eyes with keratoconjunctivitis sicca," *Archives of Ophthalmology*, vol. 101, no. 4, pp. 557-558, 1983.

[13] L. Luo, D. Q. Li, and S. C. Pflugfelder, "Hyperosmolarity-induced apoptosis in human corneal epithelial cells is mediated by cytochrome c and MAPK pathways," *Cornea*, vol. 26, no. 4, pp. 452–460, 2007.

[14] L. Oprea, A. Tiberghien, C. Creuzot-Garcher, and C. Baudouin, "Hormonal regulatory influence in tear film," *Journal Français d'Ophtalmologie*, vol. 27, no. 8, pp. 933–941, 2004.

[15] M. E. Stern, J. Gao, T. A. Schwalb et al., "Conjunctival T-cell subpopulations in Sjögren's and non-Sjögren's patients with dry eye," *Investigative Ophthalmology & Visual Science*, vol. 43, no. 8, pp. 2609–2614, 2002.

[16] M. E. Stern, R. W. Beuerman, R. I. Fox, J. Gao, A. K. Mircheff, and S. C. Pflugfelder, "The pathology of dry eye: the interaction between the ocular surface and lacrimal glands," *Cornea*, vol. 17, no. 6, pp. 584–589, 1998.

[17] S. C. Pflugfelder, "Tear dysfunction and the cornea: LXVIII Edward Jackson Memorial Lecture," *American Journal of Ophthalmology*, vol. 152, no. 6, pp. 900–909.e1, 2011, e901.

[18] A. J. Bron, C. S. de Paiva, S. K. Chauhan et al., "TFOS DEWS II pathophysiology report," *The Ocular Surface*, vol. 15, no. 3, pp. 438–510, 2017.

[19] S. Retamozo, A. Flores-Chavez, M. Consuegra-Fernandez, F. Lozano, M. Ramos-Casals, and P. Brito-Zeron, "Cytokines as therapeutic targets in primary Sjögren syndrome," *Pharmacology & Therapeutics*, vol. 184, pp. 81–97, 2018.

[20] J. J. Nair and T. P. Singh, "Sjögren's syndrome: review of the aetiology, pathophysiology & potential therapeutic interventions," *Journal of Clinical and Experimental Dentistry*, vol. 9, no. 4, pp. e584–e589, 2017.

[21] S. K. Butcher, H. Chahal, L. Nayak et al., "Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans," *Journal of Leukocyte Biology*, vol. 70, no. 6, pp. 881–886, 2001.

[22] Q. Remijsen, T. W. Kuijpers, E. Wirawan, S. Lippens, P. Vandenabeele, and T. Vanden Berghe, "Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality," *Cell Death and Differentiation*, vol. 18, no. 4, pp. 581–588, 2011.

[23] Q. Remijsen, T. V. Berghe, E. Wirawan et al., "Neutrophil extracellular trap cell death requires both autophagy and superoxide generation," *Cell Research*, vol. 21, no. 2, pp. 290–304, 2011.

[24] S. Sonawane, V. Khanolkar, A. Namavari et al., "Ocular surface extracellular DNA and nuclease activity imbalance: a new paradigm for inflammation in dry eye disease," *Investigative Ophthalmology & Visual Science*, vol. 53, no. 13, pp. 8253–8263, 2012.

[25] S. Tibrewal, Y. Ivanir, J. Sarkar et al., "Hyperosmolar stress induces neutrophil extracellular trap formation: implications for dry eye disease," *Investigative Ophthalmology & Visual Science*, vol. 55, no. 12, pp. 7961–7969, 2014.

[26] F. Bian, F. S. A. Pelegrino, J. T. Henriksson et al., "Differential effects of dexamethasone and doxycycline on inflammation and MMP production in murine alkali-burned corneas associated with dry eye," *The Ocular Surface*, vol. 14, no. 2, pp. 242–254, 2016.

[27] Y. Gao, K. Min, Y. Zhang, J. Su, M. Greenwood, and K. Gronert, "Female-specific downregulation of tissue polymorphonuclear neutrophils drives impaired regulatory T cell and amplified effector T cell responses in autoimmune dry eye disease," *Journal of Immunology*, vol. 195, no. 7, pp. 3086–3099, 2015.

[28] C. H. Wu, K. J. Li, C. L. Yu, C. Y. Tsai, and S. C. Hsieh, "Sjögren's syndrome antigen B acts as an endogenous danger molecule to induce interleukin-8 gene expression in polymorphonuclear neutrophils," *PLoS One*, vol. 10, no. 4, article e0125501, 2015.

[29] B. N. Brown, R. Londono, S. Tottey et al., "Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials," *Acta Biomaterialia*, vol. 8, no. 3, pp. 978–987, 2012.

[30] E. L. Gautier, T. Shay, J. Miller et al., "Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages," *Nature Immunology*, vol. 13, no. 11, pp. 1118–1128, 2012.

[31] F. O. Martinez and S. Gordon, "The M1 and M2 paradigm of macrophage activation: time for reassessment," *F1000Prime Reports*, vol. 6, p. 13, 2014.

[32] M. N. Manoussakis, S. Boiu, P. Korkolopoulou et al., "Rates of infiltration by macrophages and dendritic cells and expression of interleukin-18 and interleukin-12 in the chronic inflammatory lesions of Sjögren's syndrome: correlation with certain features of immune hyperactivity and factors associated with high risk of lymphoma development," *Arthritis and Rheumatism*, vol. 56, no. 12, pp. 3977–3988, 2007.

[33] M. Sisto, S. Lisi, G. Ingravallo, D. D. Lofrumento, M. D'Amore, and D. Ribatti, "Neovascularization is prominent in the chronic inflammatory lesions of Sjögren's syndrome," *International Journal of Experimental Pathology*, vol. 95, no. 2, pp. 131–137, 2014.

[34] I. C. You, T. G. Coursey, F. Bian, F. L. Barbosa, C. S. de Paiva, and S. C. Pflugfelder, "Macrophage phenotype in the ocular surface of experimental murine dry eye disease," *Archivum Immunologiae et Therapiae Experimentalis*, vol. 63, no. 4, pp. 299–304, 2015.

[35] A. Iwasa, R. Arakaki, N. Honma et al., "Aromatase controls Sjögren syndrome-like lesions through monocyte chemotactic protein-1 in target organ and adipose tissue-associated macrophages," *The American Journal of Pathology*, vol. 185, no. 1, pp. 151–161, 2015.

[36] C. S. Schaumburg, K. F. Siemasko, C. S. de Paiva et al., "Ocular surface APCs are necessary for autoreactive T cell-mediated experimental autoimmune lacrimal keratoconjunctivitis," *Journal of Immunology*, vol. 187, no. 7, pp. 3653–3662, 2011.

[37] D. Zhou, Y. T. Chen, F. Chen et al., "Critical involvement of macrophage infiltration in the development of Sjögren's syndrome-associated dry eye," *The American Journal of Pathology*, vol. 181, no. 3, pp. 753–760, 2012.

[38] V. Hauk, L. Fraccaroli, E. Grasso et al., "Monocytes from Sjögren's syndrome patients display increased vasoactive intestinal peptide receptor 2 expression and impaired apoptotic cell phagocytosis," *Clinical and Experimental Immunology*, vol. 177, no. 3, pp. 662–670, 2014.

[39] M. I. García-Laorden, I. Stroo, S. Terpstra et al., "Expression and function of granzymes A and B in *Escherichia coli* peritonitis and sepsis," *Mediators of Inflammation*, vol. 2017, Article ID 4137563, 11 pages, 2017.

[40] J. Pardo, S. Balkow, A. Anel, and M. M. Simon, "Granzymes are essential for natural killer cell-mediated and perf-facilitated tumor control," *European Journal of Immunology*, vol. 32, no. 10, pp. 2881–2886, 2002.

[41] F. Dotiwala, S. Mulik, R. B. Polidoro et al., "Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites," *Nature Medicine*, vol. 22, no. 2, pp. 210–216, 2016.

[42] Y. Li, K. To, P. Kanellakis et al., "$CD4^+$ natural killer T cells potently augment aortic root atherosclerosis by perforin- and granzyme B-dependent cytotoxicity," *Circulation Research*, vol. 116, no. 2, pp. 245–254, 2015.

[43] C. Boni, P. Lampertico, L. Talamona et al., "Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B," *Hepatology*, vol. 62, no. 6, pp. 1697–1709, 2015.

[44] P. Lemire, T. Galbas, J. Thibodeau, and M. Segura, "Natural killer cell functions during the innate immune response to pathogenic streptococci," *Frontiers in Microbiology*, vol. 8, p. 1196, 2017.

[45] W. Jiang, D. Li, R. Han et al., "Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 114, no. 30, pp. E6202–E6211, 2017.

[46] G. Ren, T. Shao, Y. Zhuang et al., "Association of killer cell immunoglobulin-like receptor and human leukocyte antigen-C genotype with dry eye disease in a Chinese Han population," *Genetic Testing and Molecular Biomarkers*, vol. 16, no. 8, pp. 910–914, 2012.

[47] Y. Chen, S. K. Chauhan, D. R. Saban, Z. Sadrai, A. Okanobo, and R. Dana, "Interferon-γ-secreting NK cells promote induction of dry eye disease," *Journal of Leukocyte Biology*, vol. 89, no. 6, pp. 965–972, 2011.

[48] X. Zhang, E. A. Volpe, N. B. Gandhi et al., "NK cells promote Th-17 mediated corneal barrier disruption in dry eye," *PLoS One*, vol. 7, no. 5, article e36822, 2012.

[49] K. C. Yoon, C. S. Park, I. C. You et al., "Expression of CXCL9, −10, −11, and CXCR3 in the tear film and ocular surface of patients with dry eye syndrome," *Investigative Ophthalmology & Visual Science*, vol. 51, no. 2, pp. 643–650, 2010.

[50] T. Bose, R. Lee, A. Hou, L. Tong, and K. G. Chandy, "Tissue resident memory T cells in the human conjunctiva and immune signatures in human dry eye disease," *Scientific Reports*, vol. 7, article 45312, 2017.

[51] J. Y. Niederkorn, M. E. Stern, S. C. Pflugfelder et al., "Desiccating stress induces T cell-mediated Sjogren's syndrome-like lacrimal keratoconjunctivitis," *Journal of Immunology*, vol. 176, no. 7, pp. 3950–3957, 2006.

[52] Y. Komiyama, S. Nakae, T. Matsuki et al., "IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis," *Journal of Immunology*, vol. 177, no. 1, pp. 566–573, 2006.

[53] S. Nakae, A. Nambu, K. Sudo, and Y. Iwakura, "Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice," *Journal of Immunology*, vol. 171, no. 11, pp. 6173–6177, 2003.

[54] W. Chi, X. Zhu, P. Yang et al., "Upregulated IL-23 and IL-17 in Behçet patients with active uveitis," *Investigative Ophthalmology & Visual Science*, vol. 49, no. 7, pp. 3058–3064, 2008.

[55] C. S. De Paiva, S. Chotikavanich, S. B. Pangelinan et al., "IL-17 disrupts corneal barrier following desiccating stress," *Mucosal Immunology*, vol. 2, no. 3, pp. 243–253, 2009.

[56] T. H. Dohlman, S. K. Chauhan, S. Kodati et al., "The CCR6/CCL20 axis mediates Th17 cell migration to the ocular surface in dry eye disease," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 6, pp. 4081–4091, 2013.

[57] T. G. Coursey, N. B. Gandhi, E. A. Volpe, S. C. Pflugfelder, and C. S. de Paiva, "Chemokine receptors CCR6 and CXCR3 are necessary for $CD4^+$ T cell mediated ocular surface disease in experimental dry eye disease," *PLoS One*, vol. 8, no. 11, article e78508, 2013.

[58] T. H. Dohlman, J. Ding, R. Dana, and S. K. Chauhan, "T cell-derived granulocyte-macrophage colony-stimulating factor contributes to dry eye disease pathogenesis by promoting CD11b+ myeloid cell maturation and migration," *Investigative Ophthalmology & Visual Science*, vol. 58, no. 2, pp. 1330–1336, 2017.

[59] B. Subbarayal, S. K. Chauhan, A. Di Zazzo, and R. Dana, "IL-17 augments B cell activation in ocular surface autoimmunity," *Journal of Immunology*, vol. 197, no. 9, pp. 3464–3470, 2016.

[60] E. Knop, N. Knop, and P. Claus, "Local production of secretory IgA in the eye-associated lymphoid tissue (EALT) of the normal human ocular surface," *Investigative Ophthalmology & Visual Science*, vol. 49, no. 6, pp. 2322–2329, 2008.

[61] G. Nocturne and X. Mariette, "B cells in the pathogenesis of primary Sjögren syndrome," *Nature Reviews Rheumatology*, vol. 14, no. 3, pp. 133–145, 2018.

[62] J. R. Smith, A. J. Stempel, A. Bharadwaj, and B. Appukuttan, "Involvement of B cells in non-infectious uveitis," *Clinical & Translational Immunology*, vol. 5, no. 2, article e63, 2016.

[63] E. Miserocchi, I. Pontikaki, G. Modorati, M. Gattinara, P. L. Meroni, and V. Gerloni, "Anti-CD 20 monoclonal antibody (rituximab) treatment for inflammatory ocular diseases," *Autoimmunity Reviews*, vol. 11, no. 1, pp. 35–39, 2011.

[64] S. Barabino, E. Montaldo, F. Solignani, C. Valente, M. C. Mingari, and M. Rolando, "Immune response in the conjunctival epithelium of patients with dry eye," *Experimental Eye Research*, vol. 91, no. 4, pp. 524–529, 2010.

[65] M. E. Stern, C. S. Schaumburg, K. F. Siemasko et al., "Autoantibodies contribute to the immunopathogenesis of experimental dry eye disease," *Investigative Ophthalmology & Visual Science*, vol. 53, no. 4, pp. 2062–2075, 2012.

[66] A. J. McClellan, E. A. Volpe, X. Zhang et al., "Ocular surface disease and dacryoadenitis in aging C57BL/6 mice," *The American Journal of Pathology*, vol. 184, no. 3, pp. 631–643, 2014.

[67] P. G. McMenamin, S. M. Morrison, and C. McMenamin, "Immunomorphologic studies of mast cell heterogeneity, location, and distribution in the rat conjunctiva," *The Journal of Allergy and Clinical Immunology*, vol. 97, no. 6, pp. 1375–1386, 1996.

[68] J. Liu, T. Fu, F. Song et al., "Mast cells participate in corneal development in mice," *Scientific Reports*, vol. 5, no. 1, article 17569, 2015.

[69] A.-M. A. Irani, "Ocular mast cells and mediators," *Immunology and Allergy Clinics of North America*, vol. 28, no. 1, pp. 25–42, 2008.

[70] A. Matsuda, Y. Okayama, N. Terai et al., "The role of interleukin-33 in chronic allergic conjunctivitis," *Investigative Ophthalmology & Visual Science*, vol. 50, no. 10, pp. 4646–4652, 2009.

[71] D. J. Royer, M. Zheng, C. D. Conrady, and D. J. J. Carr, "Granulocytes in ocular HSV-1 infection: opposing roles of mast cells and neutrophils," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 6, pp. 3763–3775, 2015.

[72] M. Hingorani, D. Metz, and S. L. Lightman, "Characterisation of the normal conjunctival leukocyte population," *Experimental Eye Research*, vol. 64, no. 6, pp. 905–912, 1997.

[73] K. Fukagawa, N. Okada, H. Fujishima et al., "Corneal and conjunctival fibroblasts are major sources of eosinophil-recruiting chemokines," *Allergology International*, vol. 58, no. 4, pp. 499–508, 2009.

[74] J. Sugita, Y. Asada, W. Ishida et al., "Contributions of Interleukin-33 and TSLP in a papain-soaked contact lens-induced mouse conjunctival inflammation model," *Immunity, Inflammation and Disease*, vol. 5, no. 4, pp. 515–525, 2017.

[75] Y. Huang, B. Yang, and W. Li, "Defining the normal core microbiome of conjunctival microbial communities," *Clinical Microbiology and Infection*, vol. 22, no. 7, pp. 643.e7–643.e12, 2016.

[76] L. J. Lu and J. Liu, "Human microbiota and ophthalmic disease," *The Yale Journal of Biology and Medicine*, vol. 89, no. 3, pp. 325–330, 2016.

[77] Z. K. Wang, Y. S. Yang, Y. Chen, J. Yuan, G. Sun, and L. H. Peng, "Intestinal microbiota pathogenesis and fecal microbiota transplantation for inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 20, no. 40, pp. 14805–14820, 2014.

Articulo 4: Oscar Illescas, Thalia Pacheco-Fernández, Juan P. Laclette, Tonathiu Rodriguez, Miriam Rodriguez-Sosa. 2020. Immune modulation by the macrophage migration inhibitory factor (MIF)family: D-dopachrome tautomerase (DDT) is not (always) a backup system. Cytokine. IF.3.4

Cytokine 133 (2020) 155121

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Review article

Immune modulation by the macrophage migration inhibitory factor (MIF) family: D-dopachrome tautomerase (DDT) is not (always) a backup system

Oscar Illescas[a], Thalia Pacheco-Fernández[a], Juan P. Laclette[b], Tonathiu Rodriguez[a], Miriam Rodriguez-Sosa[a,*]

[a] Biomedicine Unit, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), Tlalnepantla, MEX C.P. 54090, Mexico
[b] Department of Immunology, Institute of Biomedical Research, Universidad Nacional Autónoma de México (UNAM), Mexico City C.P. 04510, Mexico

ARTICLE INFO

Keywords:
Macrophage migration inhibitory factor
MIF
DDT
D-dopachrome tautomerase
CD74

ABSTRACT

Human macrophage migration inhibition factor (MIF) is a protein with cytokine and chemokine properties that regulates a diverse range of physiological functions related to innate immunity and inflammation. Most research has focused on the role of MIF in different inflammatory diseases. D-dopachrome tautomerase (DDT), a different molecule with structural similarities to MIF, which shares receptors and biological functions, has recently been reported, but little is known about its roles and mechanisms. In this review, we sought to understand the similarities and differences between these molecules by summarizing what is known about their different structures, receptors and mechanisms regulating their expression and biological activities with an emphasis on immunological aspects.

1. Introduction

MIF is a cytokine that has an important role as a modulator of innate immunity produced by the pituitary gland and many different cell types. MIF expression and release are regulated by different mechanisms in response to danger and inflammatory signals [1–4]. D-dopachrome tautomerase (DDT, also referred to as MIF-2) and DDT-like protein (DDTL) are MIF family members products of the *DDT* and *DDTL* genes, which are homologous to *MIF* [5,6]. DDT shows 34% sequence identity with MIF and close three-dimensional structural homology (Fig. 1), including the presence of the MIF superfamily canonical NH2-terminal proline. The genes encoding all MIF family members are located in the 22q11.23 locus [5], where some copy number variants have been identified [7].

Recent years have seen considerable advances have made in our understanding of the role and functions of MIF in mammalian immunity and homeostasis. Although DDT shares some structural and genetic similarities with MIF, the biological function of DDT has received little attention, even though there is growing evidence that DDT may have different roles than MIF. Therefore, the existence of different regulatory mechanisms for MIF and DDT should be explored.

Here, rather than providing an exhaustive description of each molecule, we summarize our current knowledge in a broad overview, citing examples where relevant, and specifically focus on recent discoveries concerning the DDT molecule. We subdivided this review into a brief description about the complex relationships between these two molecules, their structure, receptors, general functions, antimicrobial immunity, roles in autoimmunity and cancer, and an abstract of some redundant and nonredundant activities.

2. Protein structure

The active forms of MIF and DDT are homotrimers, each forming a barrel-like structure (Fig. 1). This quaternary structure is highly similar between both proteins and conserved among species. The third and most recently reported member of the family, DDTL, has a putative lyase activity and shares an identical portion of its primary sequence with DDT. DDTL has no reported crystal structure; however, modelling of its structure using the DDT quaternary structure as a reference is possible due to their high homology (Fig. 1). Apparently, the non-identical 23 amino acids (positions 96–118 in both sequences) and the 16 extra amino acids in the c-terminal may not interfere with the assembly of trimers.

Differences in functional motifs can be found by comparing the structures of MIF family members. MIF contains a pseudo (E)LR domain, which is essential for its chemokine function, while DDT does not. DDT also lacks the CXXC redox motif present on MIF; this motif is important in sensing redox signals by thiol-based regulators and is

* Corresponding author at: Unidad de Biomedicina, FES-Iztacala, UNAM, Av. De los Barrios 1, Los Reyes Iztacala, Tlalnepantla, Edo de México 54090, Mexico.
E-mail address: rodriguezm@unam.mx (M. Rodriguez-Sosa).

https://doi.org/10.1016/j.cyto.2020.155121
Received 9 September 2019; Received in revised form 29 April 2020; Accepted 6 May 2020
Available online 11 May 2020
1043-4666/ © 2020 Elsevier Ltd. All rights reserved.

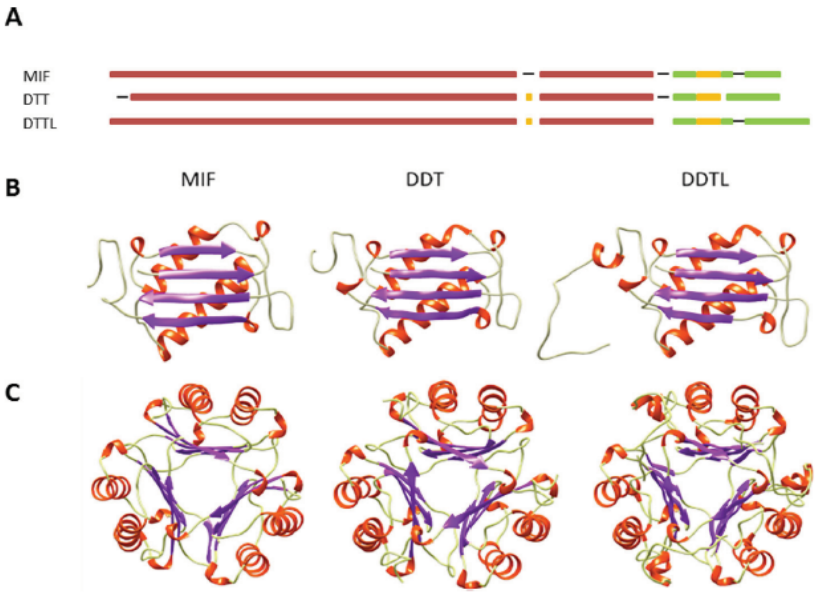


Fig. 1. MIF, DDT and DDTL. A. Comparison of MIF, DDT and DDTL amino acid sequences. Identical regions are highlighted (red), as well as high (yellow) and low similarity regions (green); lack of similarity is represented with a black line. **B.** Monomer and **C.** trimer structures of the MIF family proteins were rendered in UCSF Chimera, based on MIF and DDT crystal structures available in RCSB Protein Data Bank (PDB ID: 1MIF and 1DPT), and DDTL structure was predicted with RaptorX [11].

typical of disulfide reductases [6,8–10]. As we will further explain, these different redox and chemotactic capacities are central to understand the similarities and differences between MIF and DDT.

2.1. Post-translational modifications

Another important difference is that MIF has several post-translational modifications that produce changes on its own activity [12], while DDT has fewer modifications that have been described so far. For example, a cysteinylated MIF form mainly produced by CD4 + CD25- cells, has been linked to an inhibitory effect on IL-4 expression in CD4 + T cells and to IgE antibody response suppression *in vitro* [13,14].

MIF glycosylation produces an *O*-GlcNAcylated form, and the modified MIF is secreted and bind to EGFR, blocking EGFR ligand binding activation. In the context cancer setting, activation of EGFR is associated with ERK-1/2 and c-Jun signalling cascades and it promotes tumour progression [15]. O-GlcNAcylated MIF acts as an antitumour molecule by inhibiting this activation.

The only post-translational modification known for DDT is also shared by MIF and is the removal of the N-terminal methionine and exposure of a proline residue, which is necessary for enzymatic activity [16,17]. In MIF this residue can bind isothiocyanate, which results in loss of tautomerase activity and its capacity to bind CD74 [18,19].

2.2. Oxidized forms of MIF

Three different oxidized forms of MIF have been described. A reversible oxidized form created by covalent binding of sulfhydryl-reactive compounds to C81, which is referred to as oxMIF, results in a conformational change due to a β-sheet content reduction [20,21]. oxMIF was found to be selectively expressed under a variety of inflammatory conditions, and treatment with specific antibodies directed against this oxidized form did not affect systemic MIF levels but reduced inflammation in mouse models of chronic and acute enterocolitis [21]. Imalumab (BAX69), a recombinant human antibody directed against this oxidized form of MIF demonstrated modest antitumour activity alone or in combination with other treatments [22,23]. A different post-translational modification directed to the same residue, C81 S-nitrosylation, impedes MIF's interaction with the c-Jun activation domain-binding protein-1 (JAB1) and has demonstrated cardioprotective activity mediated by CD74 and the AMPK pathway [24]. Further research on this matter is needed considering that these modifications may either lead to similar structural modifications or be mutually exclusive.

Myeloperoxidase-derived ROS production by activated neutrophils results in oxidation of the MIF N-terminal proline to a proline imine. This second form of oxidized MIF loses its tautomerase activity, but maintain proinflammatory cytokine induction and antiapoptotic activities [25]. Interestingly, MIF and DDT are indirectly involved in important chemotactic functions for neutrophils through the activation of resident macrophages and the secretion of IL-8 [26]; however, this modified MIF without terminal proline may not be capable to stimulate the production of superoxide by neutrophils [27] or the expression of MMP-related genes [28]. This suggests a regulatory mechanism for MIF in neutrophil-related acute inflammation to limit the damage caused by ROS (Fig. 2).

Recently, oxidation of an unidentified MIF residue was reported in brains with early Alzheimer's disease. This modification impeded tautomerase activity but did not affect redox capabilities [29]. Considering DDT similarities with MIF and that all oxidized MIF forms were identified and obtained under different conditions, the possibility that DDT has an oxidized form is likely, even though DDT lacks redox capabilities, and this may comprise functional and regulatory differences between the two proteins.

The effect of post-translational modifications and oxidized forms on the MIF family activity has not been fully considered in the design of therapies based on MIF/DDT inhibitors or specific antibodies. The rationale is usually based on the canonical function of MIF alone and does not consider the consequences of blocking the other roles that both proteins possess, even those that could be beneficial to the patient.

3. Receptors

3.1. CD74 and CD44

Both MIF and DDT bind to the type II transmembrane protein CD74

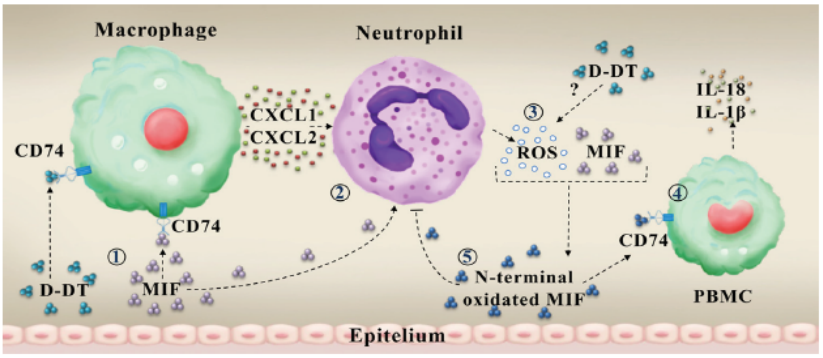


Fig. 2. MIF family neutrophil driven regulation in acute inflammation. (1) MIF and DDT are released by epithelial cells and bind the receptor CD74 on macrophages, activating the secretion of CXCL1 and CXCL2. (2) The chemokines CXCL1 and CXCL2 attract neutrophils, and MIF enhances their superoxide production. (3) Neutrophil-produced ROS oxidize MIF N-terminal proline, causing the loss of the tautomerase activity. (4) Oxidized MIF retains the ability to bind CD74 in peripheral mononuclear blood cells (PBMCs) inducing IL-1β and IL-18 secretion. (5) Oxidized MIF cannot stimulate neutrophil superoxide generation secretion.

[9,30], the plasma membrane form of the invariant chain of MHC class II. CD74 features shorts cytoplasmatic and intermembrane domains, and a long extracytoplasmic region [31].

MIF signalling through CD74 requires CD44, a glycoprotein involved in cell-to-cell interactions, adhesion and migration. MIF binds to the extracellular portion of CD74 but does not bind to CD44. After binding CD74, MIF activates the PI3K-Akt and ERK-1/2 signalling pathways [32,33]. The CD74-MIF complex can also be endocytosed through a β-arrestin-mediated and clathrin-dependent process, resulting in the sustained activation of ERK-1/2 [34], and downregulation of glucocorticoid-induced leucine zipper (GILZ) and mitogen-activated protein kinase (MAPK)-phosphatase 1 (MKP-1) expression [35–37]. GILZ and MKP-1 are intracellular proteins that inhibit targets such as NF-κB, AP1, Raf1, Ras and the MAPK ERK-1/2 pathway [35–38]. CD74 is also necessary for MIF and DDT cooperative activation of JNK pathway and c-Jun phosphorylation, which results in subsequent activation of AP1 and expression of IL-8, a chemotactic, prophagocytic and proangiogenic inflammatory factor [39].

MIF was thought to be the only ligand of CD74 until it was shown in 2011 that DDT also binds to it. DDT-CD74 complexes have different association/dissociation constants compared to those of MIF, which results in a faster and more frequent association. The binding induces internalization of CD74, although it does not always trigger a signalling cascade [9]. In some cases DDT-CD74-CD44 binding in macrophages triggers several cell-survival mechanisms, such as activation of the ERK-1/2 MAPK pathway or upregulation of NF-κB [30,40].

Expression of CD44 is independently induced by DDT and MIF [41,42], and similar to MIF, ERK-1/2 activation by DDT requires both CD74 and CD44 in macrophages [9]. However, in Simpson-Golabi-Behmel syndrome cells, a human preadipocyte cell line, it was found that DDT induces IL-6 expression through CD44-independent ERK-1/2 activation [43]. This suggests that the mechanism for ERK activation through CD74 may vary depending on the cell population.

A soluble form of CD74 (sCD74) lacking the transmembrane and intracellular regions has been recently described [44]. sCD74 is normally found in serum, macrophages secrete it after MIF stimulation, its serum levels increase after lung and skin injury, and it can bind MIF and DDT, thus blocking their interaction with cell surface CD74 [44–46]. Therefore, secretion of sCD74 could represent a self-regulatory mechanism.

CD74 is also a dormant transcription factor; it undergoes regulated intramembrane proteolysis (RIP) after MIF binding, liberating a 42 residue-long peptide (CD74-ICD) that translocates into the nucleus and activates p65, resulting in activation of antiapoptotic signals [47,48]. Although possible, it is not clear whether CD74-ICD and sCD74 are produced at the same time.

3.2. CXCR2, CXCR4 and CXCR7

MIF is considered a noncognate ligand for CXCR2 and CXCR4. It induces chemotaxis and arrest of monocytes, primary B cells and T cells under atherogenic or inflammatory conditions [8,49]. MIF also has a moderate CXCR2-dependent chemotactic effect on neutrophils even when they do not express CD74 [8], and it induces eosinophil chemotaxis through CXCR4 [50].

Hypoxia induces both DDT and MIF secretion [51,52]. The latter recruits endothelial progenitor cells (EPCs) in a CXCR4-dependent manner, and interestingly, EPCs have angiogenic and vasculogenic activity and secrete MIF, generating positive feedback [53,54]. Lysophosphatidic acid is a bioactive lipid mediator with a prominent role in cancer that induces the expression of hypoxia inducible factor-1α (HIF-1α), which in turn induces the transcription of MIF and DDT [52,55,56]. Conversely, HIF-1α is stabilized by interactions with MIF and JAB1 (Fig. 3) [55].

DDT is regulated by HIF1-α and HIF2-α in a similar fashion [52], but there is no evidence of DDT carrying out the same chemotactic activities; furthermore, DDT lacks the pseudo (E)LR domain of MIF, which is essential for its interaction with CXCR2. This may imply a fundamental difference in the roles of DDT and MIF on recruiting monocytes and leukocytes [6,8,9].

It has also been reported that MIF interacts with CXCR7, a receptor involved in cellular adhesion and proliferation involved in different diseases such as multiple sclerosis, rheumatoid arthritis, atherosclerosis and cancer [57,58]. MIF binding to CXCR7 in platelets induces activation of the PI3K-Akt pathway and inactivation of the proapoptotic protein BAD, effectively promoting cell survival. However, the binding of MIF to CXCR4 induces internalization of the latter but not activation of the ERK-1/2 pathway, which is dependent on CD74 [59]. There is no evidence of DDT binding to CXCR4 or CXCR7.

The diverse capacities of DDT and MIF to bind their receptors are the principal difference between them. DDT is a more specific ligand for CD74, as it cannot bind CXCR2 [9] nor possibly CXCR4 or CXCR7. The chemotactic activity of MIF, apparently absent in DDT, may be central to understanding their role in different diseases and should be considered when evaluating them as possible therapeutic targets or biomarkers.

3.3. Intracellular proteins

After binding CD74 and CXCR4, MIF can be endocytosed through a clathrin-dependent mechanism [60]. Once internalized, several intracellular proteins can bind MIF, including JAB1. The MIF-JAB1 complex participates in cell cycle arrest, inhibits the activation of AP1, stabilizes P27 and aids in arresting the cell in G1 phase [61,62]. JAB1 also stabilizes, and increases the activity HIF-1α [63], which in turn induces MIF gene expression (Fig. 3).

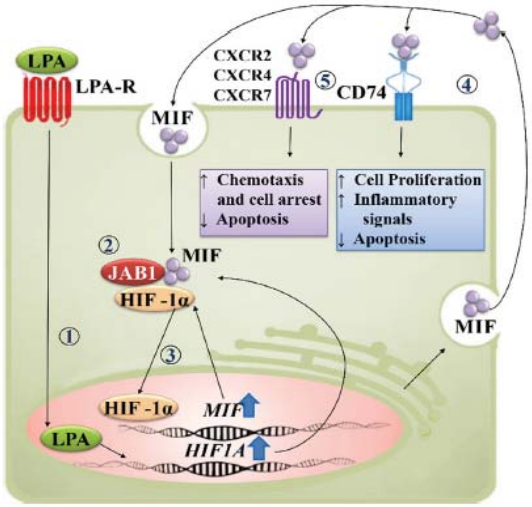


Fig. 3. MIF and HIF-1α regulation under hypoxic conditions. (1) Lysophosphatidic acid (LPA) favours the transcription of HIF-1α by increasing the binding of positive regulators to the *HIF1A* gene promoter while decreasing the binding of negative regulators (p53). (2) The transcription factor HIF-1α is bound and stabilized by internalized MIF and JAB1. (3) HIF-1α is translocated into the nucleus, inducing *MIF* and *DDT* transcription. Once MIF protein is synthesized, it can form MIF-JAB-HIF-1α complexes or be secreted. (4) Once outside the cell, MIF family binding to CD74 promotes cell proliferation, survival and expression of proinflammatory cytokines. (5) MIF induces the recruitment of monocytes, B cells, T cells and neutrophils by binding to CXCR receptors and inducing the expression of proinflammatory cytokines.

Members of the MIF family are negative regulators of the tumour suppressor p53 [64,65]. MIF and DDT cooperatively inhibit the phosphorylation, stabilization and activity of p53 in lung carcinoma [66]. However, this inhibition is reduced by the intracellular protein NME1, which binds MIF and other p53 inhibitors [67,68]. Similarly, MIF can bind and inhibit the activity of TXNIP, an intracellular inhibitor of NF-κB [69].

It is not known whether DTT also binds or is affected by any of these proteins, and binding is unlikely because DDT lacks both the CXXC and pseudo (E)LR motifs [6,9], representing another important difference between MIF family members. Additionally, the complex mutual inhibition between p53 and the MIF family, which includes several intracellular proteins and redox conditions, suggests the existence of a delicate but dynamic equilibrium between the molecules.

4. Functions

4.1. General MIF functions

MIF was originally identified as a lymphokine produced by T cells [70,71], and it was subsequently found to be secreted by a wide variety of immune and non-immune cells [72,73]. Importantly, it is constitutively expressed, stored in intracellular pools and secreted in a nonclassical manner independent of the endoplasmic reticulum and Golgi, which allows it to be rapidly released under stress conditions, inflammation and infections [73,74]. It is expressed in several cell types and released under different stimuli such as exotoxins of Gram-positive bacteria, lipopolysaccharides (LPS), hypoxic conditions, psychological stress or tissue damage [51,75–78]. Glucocorticoids induce the release of MIF by T cells [79,80], even though the proinflammatory properties of MIF antagonize the action of glucocorticoids [81] (Fig. 4). Histone acetylation and DNA methylation also regulate MIF expression. The MIF promoter is differentially hypomethylated in cells with high MIF transcription activity [82]. Also, histones associated to the MIF promoter are affected by histone deacetylase inhibitors, whose action results in the inhibition of MIF expression in a variety of cell lines [83]. Considering that all the genes of the MIF family are found in the same CpG island and locus, it is probable the same mechanisms affect the expression of DDT.

Many different roles for MIF have been demonstrated, including its functions as a proinflammatory cytokine, hormone, chemokine and activation factor of monocytes, macrophages and dendritic cells [1,85–87], and even as an enzyme with tautomerase and redox activity [88,89]. MIF also favours cell proliferation by inhibiting p53-dependent apoptosis [65] and delays cell senescence in murine embryonic fibroblasts [90].

4.2. General DDT and DDTL functions

DDT shares with MIF some catalytic and immune functions, such as counter-regulating glucocorticoid anti-inflammatory activity and triggering proinflammatory cascades by activating ERK-1/2 MAP kinases. DDT and MIF levels are increased in keratinocytes when exposed to UV rays, and at least the latter regulates melanogenesis [91–93]. DDT is also expressed constitutively, it is found at similar plasma levels to MIF in diseases such as ovarian cancer (~15 ng/mL) [9] and multiple sclerosis (~2 ng/mL) [94], and it is higher in critically ill patients (MIF = 133 vs. DDT = 292 ng/mL) [95]. This is remarkable considering that macrophages release 20 times more MIF than DDT upon LPS stimulation [9]. MIF and DDT levels also show important age-dependent variations; MIF is more abundant in foetuses (48.9 vs. 29.6 ng/mL) and 0-day-old newborns (82.6 vs. 52.0 ng/mL), while DDT is more abundant in 4-day-old newborns (109.5 vs. 121.6 ng/mL), infants between 1 and 12 months old (7.4 vs. 14.6 ng/mL) and healthy adults (5.7 vs. 7.1 ng/mL) [96,97].

Both, MIF and DDT are functional tautomerases that catalyse the conversion of D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [89], DDT also shows decarboxylase activity, converting it to 5,6-dihydroxyindole [98]. A caveat when considering the enzyme activity of both molecules is that D-dopachrome does not exist in mammals. Regardless of their similarities, MIF and DDT seem to play different, sometimes even antagonistic, roles. For example MIF and DDT have different roles in adipogenesis [99], and MIF levels are positively correlated with obesity while DDT is negatively correlated [43,100]. Therefore, the existence of different regulatory mechanisms for MIF and DDT in adipose tissue is expected. Moreover, the absence of MIF and administration of DDT increases insulin sensitivity and glucose uptake [101]. Additionally, DDT accelerates wound healing when MIF is simultaneously neutralized with antibodies [102].

Evidence suggesting the existence of the DDTL protein is scarce. RNA-seq studies have detected DDTL transcripts in many types of assayed of tissue with the highest levels in the liver, kidney and fat tissue; this is similar to DDT and in contrast to MIF, which was found to be most highly expressed in the liver, prostate and colon tissues [103].

DDT and DDTL proteins have been reported in prostatic-secreted exosomes in human urine; interestingly, they were found to be down-regulated in prostate cancer patients compared to healthy controls [104]. To our knowledge, the only other available reports of DDTL protein are proteomic studies in choroid-retinal pigment epithelium, red blood cell cytoplasm and corpus callosum of schizophrenia patients. In the last study, DDTL was even found to be differentially phosphorylated [105–107].

4.3. MIF and DDT roles in infectious diseases

MIF binding to CD74 leads to ERK phosphorylation and activation of Elk-1, a transcription factor of the Ets family that is necessary for TLR4 expression, a central molecule in the response to gram-negative

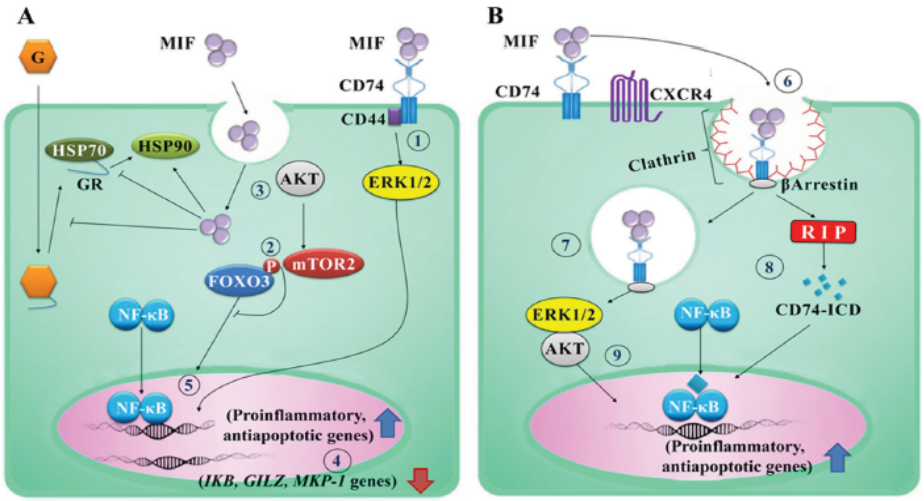


Fig. 4. Mechanisms of MIF proinflammatory and anti-glucocorticoid activities. A. Mechanisms driven by cytosolic MIF. (1) MIF binding to the cell membrane receptor CD74 in the presence of the CD44 receptor results in rapid PI3K/Akt and ERK-1/2 pathway activation. (2) Activation of the PI3K/Akt pathway leads to the phosphorylation of FOXO3, which is retained in the cytosol and/or degraded. (3) MIF competes with Glucocorticoid Receptor (GR) to bind the chaperone HSP90; GR not bound to HSP90 remains inactive and cannot bind glucocorticoids [84]. (4) Inhibition of GR and FOXO3 activity leads to downregulation of anti-inflammatory genes such as IKB, GILZ and MKP-1, (5) which in turn results in the upregulation of proinflammatory, antiapoptotic and adhesion-related genes by NF-κB and the PI3K/Akt and ERK-1/2 pathways. **B.** Mechanisms driven by MIF-CD74 complex internalization. (6) MIF can be internalized after binding to CD74 and CXCR4 through a clathrin-dependent and β-arrestin-mediated mechanism. (7) Internalization allows sustained activation of the PI3K/Akt and ERK-1/2 pathways. (8) CD74 can undergo regulated intramembrane proteolysis (RIP), liberating its intracellular domain (CD74-ICD), (9) which is translocated to the nucleus where it favours NF-κB-induced transcription.

bacteria. Coincidently, MIF has been shown to upregulate TLR4 [4,108]. This coordinated action helps activate immune cells such as macrophages, and initiates the proinflammatory response, making MIF a constitutive element of host antimicrobial defences. Therefore, MIF has been implicated in the response against gram-negative bacteria such as *Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhimurium* [4,109]. However, it is also necessary for the efficient activation of macrophages in the response to *Mycobacterium tuberculosis* [110] and parasites such as *Trypanosoma cruzi* [111], *Toxoplasma gondii* [85,112], *Leishmania major* [113] and *Taenia crassiceps* [114].

MIF does not always function in protecting the host against infection. An excessive MIF-mediated inflammatory response and recruitment of monocytes and neutrophils have been associated with liver injury in trypanosomiasis [115], increased malaria severity [116] and lung damage in infection by respiratory syncytial virus [117]. Additionally, *Leishmania* benefits from many of MIF activities: monocyte recruitment and conversion into macrophages, survival of infected macrophages and depleting protective CD4+ T cells [118,119]. Interestingly, DDT also attracts different subsets of macrophages to the site of infection, but only those attracted by MIF were found to be related to lethality in the context of polymicrobial sepsis [120]. Indeed, high levels of MIF are related to disease severity, adrenal response dysregulation and early death in gram-negative sepsis [121].

In fact, both DDT and MIF are elevated in sepsis patients, with DDT expression almost double that of MIF according to Pohl et al. (326 vs. 170 ng/mL) [95], inverse to the report by Merk et al. (55.5 vs. 111.0 ng/mL) [9]. The importance of MIF family is underscored by the fact that both molecules are found at even higher levels in non-surviving sepsis patients compared with survivors (MIF: 237 vs. 67 ng/mL, DDT: 375 vs. 336 ng/mL) [95]. Additionally, administration of anti-MIF antibodies and nanobodies mitigated lethality in murine models of endotoxaemia [73,122], and MIF knockout mice are resistant to its lethal effects [123]. Likewise, administration of MIF inhibitor (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) [124] or MIF-blocking murine and humanized antibodies confer protection against experimental sepsis [125,126].

The importance of MIF and DDT in infectious diseases depends on their modulation of the innate immune response as well as their involvement in multiple steps of pathogenesis. Diverse pathogens induce MIF expression or produce their own orthologue because they benefit from mechanisms such as MIF-induced autophagy and immune cell recruitment. Even though most reports have focused on MIF, DDT should not be ignored. Many of the mechanisms reported above are dependent on ERK, and DDT can activate this pathway. This could be crucial in cases where DDT is even more upregulated than MIF.

4.4. MIF and DDT regulatory functions on autoimmune diseases

The cell activation and proinflammatory action that make MIF a constitutive element of the host antimicrobial defences, contribute significantly to various immunopathologies as a result of excessive inflammation and autoimmunity [127,128]; such as arthritis [129,130], diabetes [131–133], systemic lupus erythematosus [134–136], autoimmune liver disease [44] and multiple sclerosis [137] among others.

Recent studies have shown that DDT is also increased in several inflammatory and autoimmunity diseases, and suggest that its expression varies in different cell types. For instance, DDT was found to be transcriptionally upregulated in rheumatic patients in peripheral blood mononuclear cells (fold change 2.4) [138]. Nevertheless, MIF is increased in synovial fluid and blood serum in these patients, but appears to be mainly produced by synovial cells [130]. Moreover, MIF and DDT levels are elevated in male patients with multiple sclerosis with the progressive disease phenotypes, while the CD74 receptor is overexpressed in female patients with high disease severity; however, the molecular mechanisms behind these sex-specific differences are yet to be described [139]. The pathogenic role of the MIF family in multiple sclerosis seems to be related to regulation of their receptors, as experiments on human B cells demonstrated the existence of a reciprocal

negative regulation of CD74 and CXCR4 expression. Blocking CD74 and consequential increase in CXCR4 expression led to augmented cell proliferation and suppressed expression of the death receptor Fas [140].

In diabetes the MIF-CD74 axis is recognized as being central for the development of autoimmune diabetes [141], MIF-deficient mice did not develop clinical signs in a streptozotocin-induced diabetes model [142]. Interestingly, MIF absence and exogenous DDT administration increase insulin sensitivity [101]. Additionally, experiments on MIF and DDT knockout mice indicate that DDT-deficient mice develop less severe autoimmune encephalomyelitis than wild-type mice and later than the MIF-deficient group, with higher activation of monocytes and memory T cells but less inflammatory cell migration [139]. In summary, these results suggest that DDT and CXCR4 do somehow interact and affect cell migration, but this is yet to be described.

MIF and DDT participation in pathogenesis is mainly attributed to their anti-glucocorticoid activity and induction of molecules such as TNF-α, IL-1β, VEGF and IL-8 [130]. These mechanisms are dependent on the regulation of transcription factors such as NURR1 and MKP1 [143] and sustained ERK-1/2 activation through MIF/CD74/CD44 receptor complex [42]. Nevertheless, eosinophils are chemotactic targets for MIF and their migration can be inhibited by CXCR4 antagonists or MIF inhibitors such as ISO-1. This process is relevant to diverse autoimmune diseases, as eosinophilic inflammation is essential for pathogenesis [50]. Importantly, this second mechanism is not shared by DDT.

Other factors that may contribute to pathogenesis are the functional polymorphisms that affect MIF expression [144–146], and have been implicated in autoimmune diseases such as rheumatoid arthritis, cystic fibrosis, multiple sclerosis and autoimmune hepatitis [137,144,147–149]. Interestingly, one of these polymorphisms affects the expression of both DDT and MIF [150].

Both MIF and DDT have a role in autoimmune diseases; however, existing evidence suggests many different mechanisms including those dependent on CD74 that can be elicited by both proteins and others expected to be differently regulated, such as those dependent on TLR4 and chemotaxis. Their shared importance is highlighted in those few studies considering both DDT and MIF: in autoimmune encephalomyelitis DDT deficiency results in diminished immune cell migration and a late disease onset compared to MIF deficiency [139] and in rheumatoid arthritis both molecules are needed for immune cells expansion and infiltration in tissue [151].

4.5. MIF and DDT activities in cancer

Two of the main functions of the MIF family are directly related to cancer development: induction of an inflammatory environment and the antiapoptotic activity [65]. Additionally, MIF family members are highly expressed by cancer cells and are involved in angiogenesis, tumour growth and metastasis [152–154]. Hence, MIF has recently been highly studied as a possible therapeutic target for many cancers. While this paper was being written, clinicaltrials.gov listed studies related to MIF-based therapy for colon and rectal carcinoma, ovarian cancer, acute myeloid leukaemia and glioblastoma.

MIF and DDT often play similar roles in cancer; for example, both induce cell survival, tumour growth and cell migration in renal cancer, but DDT is a more potent tumour inducer [52]. Both DDT and MIF can activate AMPK in non-malignant cells [100,155]; however, in non-small cell lung carcinoma they antagonize AMPK activation, and have a JNK-mediated proangiogenic role [39,156]. In pancreatic ductal adenocarcinoma MIF and DDT act together to promote ERK-1/2 and AKT phosphorylation and reduce p53 expression, hence promoting cell proliferation [157].

In colitis-associated colorectal cancer (CAC), both proteins have been shown to induce COX-2 transcription, which catalyses the synthesis of eicosanoids and prostaglandins with antiapoptotic and proangiogenic properties [158,159]. DDT induces COX-2 expression by two different mechanisms, dependently and independently of β-catenin. Moreover, COX-2 regulates DDT-dependent β-catenin stabilization, thus forming a positive regulation loop between the molecules [158].

Coincidently, MIF has been implicated in tumour growth, regulatory T cell (T-reg) proliferation and IL-2 production in a murine model of CAC induced by injecting CT26 cancer cells in MIF knockout mice [160]. In contrast, we found that MIF knockout mice develop a greater number of tumours than wild-type mice in a chemically induced CAC murine model [161]. Considering that in the first case the model uses well stablished cancer cells, while spontaneous cancer development is induced in the second, these disparate effects -if confirmed and extended in different cell types- would indicate that the role of the MIF family may differ according to the temporal progression of tumourigenesis.

MIF and DDT receptor CD74 also presents disparate effects in cancer. High expression of MIF and CD74 is correlated to aggressiveness of prostate cancer cell lines [162]. Differently, CD74 expression has been implicated in better survival in melanoma [163] as well as good prognosis in breast cancer and pleural mesothelioma patients [164,165], while MIF was negatively correlated or did not show any correlation [163,165,166]. None of the studies described above included data on DDT or the soluble form of CD74 that may antagonize MIF and DDT activities.

There have been many studies on MIF in the cancer context. This is understandable considering that many tumours are MIF producers and MIF participates in multiple tumour-related mechanisms such as inflammation, cell proliferation, angiogenesis, cell survival and chemotaxis. However, little attention has been paid to DDT, which shares many of its cancer-related activities. Furthermore, the MIF family may not always favour tumour development and is central to other cellular and immune mechanisms not related to cancer that should be considered when they are used as targets.

5. Clinical approaches

Members of the MIF family are useful as targets for small molecule inhibitors and antibodies and as prognostic or diagnostic biomarkers, but unfortunately several studies have focused only on MIF. Modified protein forms should also be considered, but their individual or collective utility is not known. Additionally, antibodies and inhibitor efficacy is highly variable; they act by different mechanisms, inhibit different MIF or DDT activities, and may target either both or only one MIF family member. Their multiple and shared or nonredundant activities (Table 1) are a major issue regarding their potential use in therapy.

5.1. Biomarkers

MIF and DDT have been proposed as biomarkers in diseases as diverse as endometriosis, systemic sclerosis, tuberculosis and Alzheimer's [94,174–176]. For example, MIF can be detected in urine and is a biomarker for kidney related diseases such as paediatric purpura nephritis [177], proliferative primary glomerulonephritis [178] and infection-derived pyelonephritis [179]. Urine MIF can also be used to identify cystitis/bladder pain syndrome patients [180], and the CXCL2/MIF-CXCR2 axis is considered a potential predictor and therapeutic target in bladder cancer [181]. Plasma MIF is used as biomarker for other cancer types such as head and neck squamous cell carcinoma and ovarian and gastric cancer [182–184]. It has been tested for CAC diagnosis in combination with carcinoembryonic antigen (CEA) with promising results [185].

Serum DDT has been proposed and tested as a prognostic biomarker in extensive skin burn and liver injury [186] and is correlated to mortality and organ damage in post-surgical, burn and sepsis patients in critical conditions [95]. MIF has also been implicated in mortality in experimental mice models [122,124–126] and patients [121,187]. In fact, MIF is central in the development of sepsis and endotoxaemia

Table 1
Differences and coincidences between DDT and MIF.

Feature/Activity/Interaction	DDT	MIF	References
CXXC and pseudo (E)LR motifs	No	Yes	[6,9,167,168]
Oxidized form	Unknown	Yes	[21,25,29]
Induced by LPS	Yes	Yes	[9,77]
Induced by UV rays in keratinocytes	Yes	Yes	[91,92]
Mediates melanogenesis	Unknown	Yes	[93]
Tautomerase activity	Yes	Yes	[89,98]
Target for HIF1-α transcription factor	Yes	Yes	[52,56]
Regulated by NF-κB	Unknown	Yes	[169]
Regulated by AMPK – FOXO1/mTOR axis	Yes	Unknown	[101]
Decarboxylase activity	Yes	No	[98]
CD74 binding	Yes	Yes	[9,30]
ERK sustained activation	Yes	Yes	[9,108]
ERK transient activation	Unknown	Yes	[170]
JNK activation (cooperative)	Yes	Yes	[39]
CXCR2 binding/related chemotactic activity	No	Yes	[8,9]
CXCR4/7 binding/related chemotactic activity	Unknown	Yes	[8,58]
JAB1/NME1/TXNIP binding	No	Yes	[61,67,69]
Inhibited by PAG product	Unknown	Yes	[171]
Inhibition of p53 (sinergically)	Yes	Yes	[65,66]
Necessary for insulin folding	Unknown	Yes	[172]
Produced by different cells in response to stimuli			[9,130,138]
Tissue-dependent abundance			[103]
Different and age-dependent plasma concentration			[96]
Opposing effects on adipocytes insulin resistance			[100,173]
Mutually interfering activities on fibroblast wound healing			[102]

[73,123], and both MIF and DDT are increased in sepsis patients [9,95].

MIF family expression increases after heart injury, especially in events of ischaemia reperfusion [188,189]. This seems to be due to tissue damage and not an inflammatory process [190] and is protective by activating AMPK pathway and controlling oxidative stress while inhibiting apoptosis and myocardial fibrosis [155,191–193]. However, DDT and MIF are difficult to categorize as favourable or unfavourable cardiac prognostic markers. High DDT levels have been correlated with survival of heart failure patients but also with organ dysfunction [192,194]. For MIF, high plasma levels are related to poorer recovery and lower survival of patients with infarction but are also inversely related to posterior adverse events [195,196].

MIF family members may be useful as biomarkers in diverse contexts, but the presence of sCD74 and post-translational modifications should be considered. Levels of the MIF/DDT-inhibiting sCD74 are elevated in the context of tissue injury [45,186,194], while activity modifiers such as MIF S-nitrosylation or oxidation have been identified in ischaemia reperfusion [24], neurodegeneration [29,197] and cancer [198].

High levels of MIF family members may also be indicative for personalized therapies. Nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids are widely used and both MIF and DDT antagonize their action [81]. Additionally, MIF is the target of different inhibitors and antibodies. Evaluation of MIF family levels may be used for fine-tuning these therapies or assessing their viability.

5.2. Antibodies and inhibitors

Current clinical studies targeting MIF family include specific antibodies and small molecule inhibitors [146]. Monoclonal oxMIF-specific antibody Imalumab is the only MIF family-directed antibody with demonstrated safety, although with modest effectivity [22,23]. However, MIF is also likely responsible for the effect of humanized recombinant anti-CD74 antibody Milatuzumab (hLL1) [199,200]. Phase I clinical trials showed that this antibody is well tolerated alone or in combination with other treatments, and had positive effects in refractory multiple myeloma, B-cell non-Hodgkin lymphoma and chronic lymphocytic leukaemia [201–203]. Importantly, Milatuzumab may affect all CD74-mediated mechanisms of both DDT and MIF, while Imalumab activity is limited to a single modified form of MIF.

Small molecule inhibitors have recently been actively studied [204]. Ouertatani-Sakouhi et al. [205] divided MIF inhibitors into three classes depending on their mechanism of action: (1) covalent modifiers of the catalytic site at the N-terminal proline residue, (2) molecules that noncovalently bind the catalytic site and (3) disrupters of the trimeric structure of MIF.

Class 1 and 2 inhibitors impede both enzymatic and anti-glucocorticoid MIF activities [205], but modification of the N-terminal proline by the class 1 inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) also results in partial loss of the chemotactic recruitment activity of MIF and DDT in lungs [26]; however, it is not known if all class 1 inhibitors induce this effect.

Three anti-inflammatory drugs already tested in clinical trials were found to bind and inhibit MIF, 2-phenyl-1,2-benzoselenazol-3-one (ebselen) [205], 2-methyl-1-[2-(propan-2-yl)pyrazolo[1,5-a]pyridin-3-yl]propan-1-one (ibudilast) [206] and N-(7-(Methylsulfonamido)-4-oxo-6-phenoxy-4H-chromen-3-yl)formamide (iguratimod) [207]. Ebselen is a neuroprotective agent for patients with aneurysmal subarachnoid hemorrhages and the only known member of the third inhibitor class, which disrupts MIF trimers and induces aggregation through cysteine residues. Trimer dissociation inhibits the catalytic and ligand-binding activities of MIF, strongly favouring its chemotactic activity and suggesting that trimers are not required for chemotaxis [205,208]. Iguratimod is used to treat rheumatoid arthritis. It binds MIF through an unknown mechanism and inhibits MIF tautomerase activity, but has no apparent effect on MIF knockout mice, therefore it probably cannot bind DDT [207,209]. Ibudilast is used to treat asthma and allergic conjunctivitis and has been shown to be effective in progressive multiple sclerosis. It binds non-competitively to an allosteric site near the tautomerase active site, resulting in decreased chemotactic and enzymatic activity [206,210].

The only known DDT specific inhibitor, 4-(3-carboxyphenyl)-2,5-pyridinedicarboxylic acid (4-CPPC), acts by binding the DDT c-terminal and changing its conformation in a reversible fashion, inhibiting the binding and activation of CD74 [46,211]. Importantly, the 4-CPPC and ibudilast mechanisms are different than those described for the three classes described above.

Class 1 and 2 MIF inhibitors target the tautomerase active site and effectively interfere with MIF induction of the inflammatory response [205]. However, it is not clear whether any MIF and DDT functions are dependent on their tautomerase activity. A tautomerase-inactive MIF knock-in mouse model revealed no alterations in growth regulation [212], the loss of enzymatic activity by oxidation did not alter its immunomodulatory functions [25], and the class 2 MIF inhibitor ISO-1 does not affect MIF's role in insulin folding [172]. These antecedents suggest that the ability to block receptor binding instead of tautomerase activity would better define these inhibitors.

Inhibitors have been tested in diverse disease models. ISO-1 increases survival in experimental murine sepsis [124], greatly inhibits LPS-induced proliferation in gastric cancer cells [213], suppressed tumour growth in the presence of IFN-γ in a murine melanoma model [166] and decreases the severity of murine autoimmune encephalomyelitis [214]. 4-IPP, a class 1 inhibitor of both MIF and DDT [26,215], inhibits proliferation and induces apoptosis and mitotic cell death in thyroid carcinoma [216], and sensitizes multiple myeloma cells to chemotherapy *in vitro* [217]. Ebselen has been used in cancer therapy for its ability to protect against cisplatin-induced nephrotoxicity [218]; however, it also suppresses invasion of pancreatic and renal cancer cell lines *in vitro* [219].

A different and insufficiently explored approach is agonists, which showed promising results in protection against tissue injury in a ischaemia reperfusion mouse model [220]. However, these agonists

were developed and tested before evidence suggesting that DDT also has protective potential was published [188,192], and no DDT-specific agonist has been reported.

Antibodies and small molecule inhibitors directed at the MIF family have great potential for clinic use, as the correct selection and dosage may modulate any or all activities of the family, and have corresponding risks and benefits.

6. Conclusion

The MIF family members and its receptors are affected by multiple biochemical regulatory mechanisms, but they also participate in many processes including inflammation, cell chemotaxis, proliferation and survival, and angiogenesis. As described above, MIF and DDT share several activities and may possibly back up each other when inhibited or downregulated, but this has led to a research bias. MIF remains the most studied, and DDT is often omitted even when activity differences have been identified by the few studies considering both proteins. Additionally, they are expressed and secreted by different cell types in response to stimuli.

Added complexity stems from the existence of post-translationally modified MIF species with altered activities and from the regulatory mechanisms affecting DDT that may or may not affect MIF and *vice versa*. Current therapeutic, diagnostic and prognostic use of MIF does not fully consider these intricate relationships. Prior to clinical use of inhibitors and antibodies, the entire MIF family and receptors should be considered as blocking a single mechanism may be ideal for certain diseases or insufficient for others, while abrogating all activities can be hazardous.

The known mechanisms of the MIF family should be further explored to fully understand the differences between these proteins. DDT is a more specific CD74 ligand than MIF, with fewer non-related activities. This does not make DDT less important; it implies that DDT may be a safer target for certain therapies, and that therapies directed at MIF-exclusive activities should ideally not inadvertently affect DDT. MIF has great potential in clinical research, but the complete MIF family may have greater potential.

Credit authorship contribution statement

Oscar Illescas: Writing - original draft. **Thalia Pacheco-Fernández:** Writing - review & editing. **Juan P. Laclette:** Writing - review & editing. **Tonathiu Rodriguez:** Visualization. **Miriam Rodriguez-Sosa:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported in part by grants from the "Support Program for Research Projects and Technological Innovation" UNAM-DGAPA-PAPIIT, IN-209718 and National Council of Science and Technology of Mexico (CONACYT) [A1-S-10463]. Oscar Illescas was recipient of a postdoctoral fellowship from Programa de Becas Posdoctorales DGAPA–UNAM during the realization of this review and is current holder of a postdoctoral fellowship from CONACYT. Thalia Pacheco-Fernández is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received a fellowship (CVU number 694650) from CONACYT.

References

[1] R. Bucala, MIF rediscovered: cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response, FASEB J. 10 (14) (1996) 1607–1613.
[2] D. Rosado Jde, M. Rodriguez-Sosa, Macrophage migration inhibitory factor (MIF): a key player in protozoan infections, Int J Biol Sci 7 (9) (2011) 1239–1256.
[3] T. Calandra, T. Roger, Macrophage migration inhibitory factor: a regulator of innate immunity, Nat. Rev. Immunol. 3 (10) (2003) 791–800.
[4] T. Roger, et al., MIF regulates innate immune responses through modulation of Toll-like receptor 4, Nature 414 (6866) (2001) 920.
[5] M. Coggan, et al., Structure and organization of the human theta-class glutathione S-transferase and D-dopachrome tautomerase gene complex, Biochem. J 334 (3) (1998) 617–623.
[6] N. Esumi, et al., Conserved gene structure and genomic linkage for D-dopachrome tautomerase (DDT) and MIF, Mamm. Genome 9 (9) (1998) 753–757.
[7] N. Hiroi, et al., Copy number variation at 22q11. 2: from rare variants to common mechanisms of developmental neuropsychiatric disorders, Mol. Psychiatry 18 (11) (2013) 1153–1165.
[8] J. Bernhagen, et al., MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment, Nat. Med. 13 (5) (2007) 587–596.
[9] M. Merk, et al., The D-dopachrome tautomerase (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF), Proc. Natl. Acad. Sci. 108 (34) (2011) E577–E585.
[10] M. Thiele, J. Bernhagen, Link between macrophage migration inhibitory factor and cellular redox regulation, Antioxid. Redox Signal. 7 (9–10) (2005) 1234–1248.
[11] M. Källberg, et al., Template-based protein structure modeling using the RaptorX web server, Nat. Protoc. 7 (8) (2012) 1511.
[12] L. Schindler, et al., Post-translational regulation of macrophage migration inhibitory factor: Basis for functional fine-tuning, Redox Biol. 15 (2018) 135–142.
[13] H. Watarai, et al., Posttranslational modification of the glycosylation inhibiting factor (GIF) gene product generates bioactive GIF, Proc. Natl. Acad. Sci. 97 (24) (2000) 13251–13256.
[14] M. Kim-Saijo, E.M. Janssen, K. Sugie, CD4 cell-secreted, posttranslationally modified cytokine GIF suppresses Th2 responses by inhibiting the initiation of IL-4 production, Proc. Natl. Acad. Sci. 105 (49) (2008) 19402–19407.
[15] Y. Zheng, et al., Secreted and O-GlcNAcylated MIF binds to the human EGF receptor and inhibits its activation, Nat. Cell Biol. 17 (10) (2015) 1348–1355.
[16] S.L. Stamps, M.C. Fitzgerald, C.P. Whitman, Characterization of the role of the amino-terminal proline in the enzymatic activity catalyzed by macrophage migration inhibitory factor, Biochemistry 37 (28) (1998) 10195–10202.
[17] J. Nishihira, et al., Molecular cloning of humand-dopachrome tautomerase cDNA: N-terminal proline is essential for enzyme activation, Biochem. Biophys. Res. Commun. 243 (2) (1998) 538–544.
[18] H. Ouertatani-Sakouhi, et al., A new class of isothiocyanate-based irreversible inhibitors of macrophage migration inhibitory factor, Biochemistry 48 (41) (2009) 9858–9870.
[19] K.K. Brown, et al., Direct modification of the proinflammatory cytokine macrophage migration inhibitory factor by dietary isothiocyanates, J. Biol. Chem. 284 (47) (2009) 32425–32433.
[20] A. Schinagl, et al., Role of the cysteine 81 residue of macrophage migration inhibitory factor as a molecular redox switch, Biochemistry 57 (9) (2018) 1523–1532.
[21] M. Thiele, et al., Selective targeting of a disease-related conformational isoform of macrophage migration inhibitory factor ameliorates inflammatory conditions, J. Immunol. 195 (5) (2015) 2343–2352.
[22] D. Mahalingam, et al., PD-011 Safety and efficacy analysis of imalumab, an anti-oxidized macrophage migration inhibitory factor (oxMIF) antibody, alone or in combination with 5-fluorouracil/leucovorin (5-FU/LV) or panitumumab, in patients with metastatic colorectal cancer (mCRC), Ann. Oncol. 27 (suppl_2) (2016) p. ii105–ii105.
[23] D. Mahalingam, et al., Phase I study of Imalumab (BAX69), a fully human recombinant anti-oxidized macrophage migration inhibitory factor antibody in advanced solid tumors, Br. J. Clin. Pharmacol. (2020).
[24] P. Luedike, et al., Cardioprotection through S-nitros (yl) ation of macrophage migration inhibitory factor, Circulation 125 (15) (2012) 1880–1889.
[25] N. Dickerhof, et al., Macrophage migration inhibitory factor (MIF) is rendered enzymatically inactive by myeloperoxidase-derived oxidants but retains its immunomodulatory function, Free Radical Biol. Med. 89 (2015) 498–511.
[26] D. Rajasekaran, et al., Targeting distinct tautomerase sites of D-DT and MIF with a single molecule for inhibition of neutrophil lung recruitment, FASEB J. 28 (11) (2014) 4961–4971.
[27] M. Swope, et al., Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor, EMBO J. 17 (13) (1998) 3534–3541.
[28] S. Onodera, et al., Macrophage migration inhibitory factor up-regulates expression of matrix metalloproteinases in synovial fibroblasts of rheumatoid arthritis, J. Biol. Chem. 275 (1) (2000) 444–450.
[29] O. Kassaar, et al., Macrophage migration inhibitory factor is subjected to glucose modification and oxidation in Alzheimer's disease, Sci. Rep. 7 (2017) 42874.
[30] L. Leng, et al., MIF signal transduction initiated by binding to CD74, J. Exp. Med. 197 (11) (2003) 1467–1476.
[31] M. Strubin, B. Mach, E. Long, The complete sequence of the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity, EMBO J. 3 (4) (1984) 869–872.
[32] X. Shi, et al., CD44 is the signaling component of the macrophage migration

inhibitory factor-CD74 receptor complex, Immunity 25 (4) (2006) 595–606.
[33] H. Lue, et al., Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity, Oncogene 26 (35) (2007) 5046–5059.
[34] L. Xie, et al., β-Arrestin1 mediates the endocytosis and functions of macrophage migration inhibitory factor, PLoS ONE 6 (1) (2011) e16428.
[35] T. Roger, et al., Macrophage migration inhibitory factor promotes innate immune responses by suppressing glucocorticoid-induced expression of mitogen-activated protein kinase phosphatase-1, Eur. J. Immunol. 35 (12) (2005) 3405–3413.
[36] D. Aeberli, et al., Endogenous macrophage migration inhibitory factor modulates glucocorticoid sensitivity in macrophages via effects on MAP kinase phosphatase-1 and p38 MAP kinase, FEBS Lett. 580 (3) (2006) 974–981.
[37] H. Fan, et al., Macrophage migration inhibitory factor inhibits the antiinflammatory effects of glucocorticoids via glucocorticoid-induced leucine zipper, Arthrit. Rheumatol. 66 (8) (2014) 2059–2070.
[38] E. Ayroldi, et al., GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling, J. Clin. Investig. 117 (6) (2007) 1605–1615.
[39] A.M. Coleman, et al., Cooperative regulation of non-small cell lung carcinoma angiogenic potential by macrophage migration inhibitory factor and its homolog D-dopachrome tautomerase, J. Immunol. 181 (4) (2008) 2330–2337.
[40] D. Starlets, et al., Cell-surface CD74 initiates a signaling cascade leading to cell proliferation and survival, Blood 107 (12) (2006) 4807–4816.
[41] A. Ochi, et al., MIF-2/D-DT enhances proximal tubular cell regeneration through SLPI and ATF4-dependent mechanisms, Am. J. Physiol.-Renal Physiol. (2017) p. ajprenal. 00683.2016.
[42] S.-A. Yoo, et al., MIF allele-dependent regulation of the MIF coreceptor CD44 and role in rheumatoid arthritis, Proc. Natl. Acad. Sci. 113 (49) (2016) E7917–E7926.
[43] K. Ishimoto, et al., D-dopachrome tautomerase promotes IL-6 expression and inhibits adipogenesis in preadipocytes, Cytokine 60 (3) (2012) 772–777.
[44] D.N. Assis, et al., The role of macrophage migration inhibitory factor in autoimmune liver disease, Hepatology 59 (2) (2014) 580–591.
[45] G. Wu, et al., Relationship between elevated soluble CD74 and severity of experimental and clinical ALI/ARDS, Sci. Rep. (2016) 6.
[46] P.V. Tilstam, et al., A selective small-molecule inhibitor of macrophage migration inhibitory factor-2 (MIF-2), a MIF cytokine superfamily member, inhibits MIF-2 biological activity, J. Biol. Chem. 294 (49) (2019) 18522–18531.
[47] S. Becker-Herman, et al., CD74 is a member of the regulated intramembrane proteolysis-processed protein family, Mol. Biol. Cell 16 (11) (2005) 5061–5069.
[48] I. Binsky, et al., IL-8 secreted in a macrophage migration-inhibitory factor-and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival, Proc. Natl. Acad. Sci. 104 (33) (2007) 13408–13413.
[49] **S. Tillmann, Jürgen Bernhagen, H. Noels, Arrest functions of the MIF ligand/receptor axes in atherogenesis, Front. Immunol. 4 (2013),** https://doi.org/10.3389/fimmu.2013.00115.
[50] H. De Souza, et al., Macrophage migration inhibitory factor promotes eosinophil accumulation and tissue remodeling in eosinophilic esophagitis, Mucosal Immunol. 8 (5) (2015) 1154.
[51] M. Takahashi, et al., Macrophage migration inhibitory factor as a redox-sensitive cytokine in cardiac myocytes, Cardiovasc. Res. 52 (3) (2001) 438–445.
[52] V. Pasupuleti, et al., Dysregulated D-dopachrome tautomerase, a hypoxia-inducible factor-dependent gene, cooperates with macrophage migration inhibitory factor in renal tumorigenesis, J. Biol. Chem. 289 (6) (2014) 3713–3723.
[53] D. Simons, et al., Hypoxia-induced endothelial secretion of macrophage migration inhibitory factor and role in endothelial progenitor cell recruitment, J. Cell Mol. Med. 15 (3) (2011) 668–678.
[54] C. Kupatt, et al., Embryonic endothelial progenitor cells expressing a broad range of proangiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia, FASEB J. 19 (11) (2005) 1576–1578.
[55] Y.R. No, et al., HIF1α-induced by lysophosphatidic acid is stabilized via interaction with MIF and CSN5, PLoS ONE 10 (9) (2015) e0137513.
[56] J.A. Baugh, et al., Dual regulation of macrophage migration inhibitory factor (MIF) expression in hypoxia by CREB and HIF-1, Biochem. Biophys. Res. Commun. 347 (4) (2006) 895–903.
[57] S. Alampour-Rajabi, et al., MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis, FASEB J. 29 (11) (2015) 4497–4511.
[58] M. Tarnowski, et al., Macrophage migration inhibitory factor is secreted by rhabdomyosarcoma cells, modulates tumor metastasis by binding to CXCR4 and CXCR7 receptors and inhibits recruitment of cancer-associated fibroblasts, Mol. Cancer Res. 8 (10) (2010) 1328–1343.
[59] M. Chatterjee, et al., Macrophage migration inhibitory factor limits activation-induced apoptosis of platelets via CXCR7-dependent Akt signaling, Circ. Res. 115 (11) (2014) 939–949.
[60] V. Schwartz, et al., Role for CD74 and CXCR4 in clathrin-dependent endocytosis of the cytokine MIF, Eur. J. Cell Biol. 91 (6) (2012) 435–449.
[61] R. Kleemann, et al., Intracellular action of the cytokine MIF to modulate AP-1 activity and the cell cycle through Jab1, Nature 408 (6809) (2000) 211–216.
[62] M.T. Nguyen, et al., A 16-residue peptide fragment of macrophage migration inhibitory factor, MIF-(50–65), exhibits redox activity and has MIF-like biological functions, J. Biol. Chem. 278 (36) (2003) 33654–33671.
[63] M.-K. Bae, et al., Jab1 interacts directly with HIF-1α and regulates its stability, J. Biol. Chem. 277 (1) (2002) 9–12.
[64] S.E. Brock, et al., MIF family members cooperatively inhibit p53 expression and activity, PLoS ONE 9 (6) (2014) e99795.
[65] J.D. Hudson, et al., A proinflammatory cytokine inhibits p53 tumor suppressor activity, J. Exp. Med. 190 (10) (1999) 1375–1382.
[66] S.E. Brock, et al., MIF family members cooperatively inhibit p53 expression and activity, PLoS ONE 9 (6) (2014).
[67] H. Jung, H.-A. Seong, H. Ha, Direct interaction between NM23-H1 and macrophage migration inhibitory factor (MIF) is critical for alleviation of MIF-mediated suppression of p53 activity, J. Biol. Chem. 283 (47) (2008) 32669–32679.
[68] H. Jung, H.-A. Seong, H. Ha, NM23-H1 tumor suppressor and its interacting partner STRAP activate p53 function, J. Biol. Chem. 282 (48) (2007) 35293–35307.
[69] M.J. Kim, et al., Macrophage migration inhibitory factor interacts with thioredoxin-interacting protein and induces NF-κB activity, Cell. Signal. 34 (2017) 110–120.
[70] J.R. David, Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction, Proc. Natl. Acad. Sci. 56 (1) (1966) 72–77.
[71] B.R. Bloom, B. Bennett, Mechanism of a reaction in vitro associated with delayed-type hypersensitivity, Science 153 (3731) (1966) 80–82.
[72] M. Bacher, et al., Migration inhibitory factor expression in experimentally induced endotoxemia, Am. J. Pathol. 150 (1) (1997) 235.
[73] J. Bernhagen, et al., MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia, Nature 365 (6448) (1993) 756.
[74] O. Flieger, et al., Regulated secretion of macrophage migration inhibitory factor is mediated by a non-classical pathway involving an ABC transporter, FEBS Lett. 551 (1–3) (2003) 78–86.
[75] S.V. Petrinović, et al., Macrophage migration inhibitory factor is an endogenous regulator of stress-induced extramedullary erythropoiesis, Histochem. Cell Biol. 146 (3) (2016) 311–324.
[76] T. Yoshimoto, et al., Induction of macrophage migration inhibitory factor messenger ribonucleic acid in rat forebrain by reperfusion, Neurosurgery 41 (3) (1997) 648–653.
[77] S. Salvin, J. Youngner, W. Lederer, Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity, Infect. Immun. 7 (1) (1973) 68–75.
[78] T. Calandra, et al., Macrophage migration inhibitory factor is a critical mediator of the activation of immune cells by exotoxins of Gram-positive bacteria, Proc. Natl. Acad. Sci. 95 (19) (1998) 11383–11388.
[79] M. Bacher, et al., An essential regulatory role for macrophage migration inhibitory factor in T-cell activation, Proc. Natl. Acad. Sci. 93 (15) (1996) 7849–7854.
[80] T. Calandra, et al., MIF as a glucocorticoid-induced modulator of cytokine production, Nature 377 (6544) (1995) 68–71.
[81] T. Calandra, R. Bucala, Macrophage migration inhibitory factor (MIF): a glucocorticoid counter-regulator within the immune system, Crit. Rev.™ Immunol. 17 (1) (1997).
[82] T. Roger, et [redacted] macrophage [redacted] 37 (12) (2007) 3509–3521.
[83] J. Lugrin, et al., Histone deacetylase inhibitors repress macrophage migration inhibitory factor (MIF) expression by targeting MIF gene transcription through a local chromatin deacetylation, Biochimica et Biophysica Acta (BBA)-Mol. Cell Res. 1793 (11) (2009) 1749–1758.
[84] L. Jing, M. Bu, Role of macrophage migration inhibitory factor in glucocorticoid release and glucocorticoid receptor function in rats, Ann. Clin. Lab. Sci. 41 (1) (2011) 14–19.
[85] **J.de.D. Ruiz-Rosado, J.E. Olguín, I. Juárez-Avelar, R. Saavedra, L.I. Terrazas, F.H. Robledo-Avila, A. Vazquez-Mendoza, J. Fernández, A.R. Satoskar, S. Partida-Sánchez, M. Rodriguez-Sosa, MIF promotes classical activation and conversion of inflammatory Ly6C high monocytes into TipDCs during murine toxoplasmosis, Mediators Inflamm. 2016 (2016) 1–18,** https://doi.org/10.1155/2016/9101762.
[86] C. Klasen, et al., MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling, J. Immunol. 192 (11) (2014) 5273–5284.
[87] H. Murakami, et al., Macrophage migration inhibitory factor activates antigen-presenting dendritic cells and induces inflammatory cytokines in ulcerative colitis, Clin. Exp. Immunol. 128 (3) (2002) 504–510.
[88] R. Kleemann, et al., Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase, J. Mol. Biol. 280 (1) (1998) 85–102.
[89] E. Rosengren, et al., The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction, Mol. Med. 2 (1) (1996) 143–149.
[90] S.M. Welford, et al., HIF1α delays premature senescence through the activation of MIF, Genes Dev. 20 (24) (2006) 3366–3371.
[91] B. Sonesson, et al., UVB-induced inflammation gives increased d-dopachrome tautomerase activity in blister fluid which correlates with macrophage migration inhibitory factor, Exp. Dermatol. 12 (3) (2003) 278–282.
[92] T. Shimizu, et al., Ultraviolet B radiation upregulates the production of macrophage migration inhibitory factor (MIF) in human epidermal keratinocytes, J, Invest. Dermatol. 112 (2) (1999) 210–215.
[93] A. Enomoto, et al., UV-B radiation induces macrophage migration inhibitory factor-mediated melanogenesis through activation of protease-activated receptor-2 and stem cell factor in keratinocytes, Am. J. Pathol. 178 (2) (2011) 679–687.
[94] F.B. Vincent, et al., Analysis of serum macrophage migration inhibitory factor and D-dopachrome tautomerase in systemic sclerosis, Clin. Transl. Immunol. 7 (12) (2018) e1042.
[95] J. Pohl, et al., Elevated MIF-2 levels predict mortality in critically ill patients, J. Crit. Care 40 (2017) 52–57.
[96] T. Roger, et al., Plasma levels of macrophage migration inhibitory factor and D-dopachrome tautomerase show a highly specific profile in early life, Front.

http://refhub.elsevier.com/S1043-46

Immunol. 8 (2017) 26.
[97] T. Roger, et al., High expression levels of macrophage migration inhibitory factor sustain the innate immune responses of neonates, Proc. Natl. Acad. Sci. 113 (8) (2016) E997–E1005.
[98] P. Björk, et al., A new enzyme activity in human blood cells and isolation of the responsible protein (d-dopachrome tautomerase) from erythrocytes, Eur. J. Haematol. 57 (3) (1996) 254–256.
[99] B.-S. Kim, et al., The macrophage migration inhibitory factor protein superfamily in obesity and wound repair, Exp. Mol. Med. 47 (5) (2015) e161.
[100] T. Iwata, et al., The action of D-dopachrome tautomerase as an adipokine in adipocyte lipid metabolism, PLoS ONE 7 (3) (2012) e33402.
[101] T. Iwata, et al., The AMPK/mTOR pathway is involved in D-dopachrome tautomerase gene transcription in adipocytes differentiated from SGBS cells, a human preadipocyte cell line, Cytokine 96 (2017) 195–202.
[102] B.S. Kim, et al., D-dopachrome tautomerase in adipose tissue inflammation and wound repair, J. Cell Mol. Med. 21 (1) (2017) 35–45.
[103] L. Fagerberg, et al., Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics, Mol. Cell. Proteom. 13 (2) (2014) 397–406.
[104] S. Principe, et al., In-depth proteomic analyses of exosomes isolated from expressed prostatic secretions in urine, Proteomics 13 (10–11) (2013) 1667–1671.
[105] M. Dammalli, et al., Toward postgenomics ophthalmology: a proteomic map of the human choroid-retinal pigment epithelium tissue, OMICS: J. Integr. Biol. 21 (2) (2017) 114–122.
[106] V.M. Saia-Cereda, et al., Differential proteome and phosphoproteome may impact cell signaling in the corpus callosum of schizophrenia patients, Schizophr. Res. 177 (1) (2016) 70–77.
[107] M. Masseroli, et al., Searching for specific motifs in affinity capture in proteome analysis, J. Proteom. 72 (5) (2009) 791–802.
[108] R.A. Mitchell, et al., Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF) Regulatory role in cell proliferation and glucocorticoid action, J. Biol. Chem. 274 (25) (1999) 18100–18106.
[109] H. Koebernick, et al., Macrophage migration inhibitory factor (MIF) plays a pivotal role in immunity against Salmonella typhimurium, Proc. Natl. Acad. Sci. 99 (21) (2002) 13681–13686.
[110] R. Das, et al., Macrophage migration inhibitory factor (MIF) is a critical mediator of the innate immune response to Mycobacterium tuberculosis, Proc. Natl. Acad. Sci. 110 (32) (2013) E2997–E3006.
[111] R.A. Cutrullis, P.B. Petray, R.S. Corral, MIF-driven activation of macrophages induces killing of intracellular Trypanosoma cruzi dependent on endogenous production of tumor necrosis factor, nitric oxide and reactive oxygen species, Immunobiology 222 (2) (2017) 423–431.
[112] M. Flores, et al., Macrophage migration inhibitory factor (MIF) is critical for the host resistance against Toxoplasma gondii, FASEB J. 22 (10) (2008) 3661–3671.
[113] A.R. Satoskar, et al., Migration-inhibitory factor gene-deficient mice are susceptible to cutaneous Leishmania major infection, Infect. Immun. 69 (2) (2001) 906–911.
[114] M. Rodriguez-Sosa, et al., Macrophage migration inhibitory factor plays a critical role in mediating protection against the helminth parasite Taenia crassiceps, Infect. Immun. 71 (3) (2003) 1247–1254.
[115] B. Stijlemans, et al., MIF contributes to Trypanosoma brucei associated immunopathogenicity development, PLoS Pathog. 10 (9) (2014) e1004414.
[116] C. Han, et al., Plasma concentration of malaria parasite-derived macrophage migration inhibitory factor in uncomplicated malaria patients correlates with parasitemia and disease severity, Clin. Vaccine Immunol. 17 (10) (2010) 1524–1532.
[117] G.F. de Souza, et al., Macrophage migration inhibitory factor (MIF) controls cytokine release during respiratory syncytial virus infection in macrophages, Inflamm. Res. 68 (6) (2019) 481–491.
[118] D. Kamir, et al., A Leishmania ortholog of macrophage migration inhibitory factor modulates host macrophage responses, J. Immunol. 180 (12) (2008) 8250–8261.
[119] T. Holowka, et al., Leishmania-encoded orthologs of macrophage migration inhibitory factor regulate host immunity to promote parasite persistence, FASEB J. 30 (6) (2016) 2249–2265.
[120] P.V. Tilstam, et al., Selective recruitment of lethal pro-inflammatory macrophages in sepsis by MIF but not D-DT (MIF-2), J. Immunol. 202 (1 Supplement) (2019) 51.9.
[121] M. Emonts, et al., Association between high levels of blood macrophage migration inhibitory factor, inappropriate adrenal response, and early death in patients with severe sepsis, Clin. Infect. Dis. 44 (10) (2007) 1321–1328.
[122] A. Sparkes, et al., Novel half-life extended anti-MIF nanobodies protect against endotoxic shock, FASEB J. 32 (6) (2018) 3411–3422.
[123] M. Bozza, et al., Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis, J. Exp. Med. 189 (2) (1999) 341–346.
[124] Y. Al-Abed, et al., ISO-1 binding to the tautomerase active site of MIF inhibits its pro-inflammatory activity and increases survival in severe sepsis, J. Biol. Chem. 280 (44) (2005) 36541–36544.
[125] T. Calandra, et al., Protection from septic shock by neutralization of macrophage migration inhibitory factor, Nat. Med. 6 (2) (2000) 164–170.
[126] R.J. Kerschbaumer, et al., Neutralization of macrophage migration inhibitory factor (MIF) by fully human antibodies correlates with their specificity for the β-sheet structure of MIF, J. Biol. Chem. 287 (10) (2012) 7446–7455.
[127] S. Stosic-Grujicic, I. Stojanovic, F. Nicoletti, MIF in autoimmunity and novel therapeutic approaches, Autoimmun. Rev. 8 (3) (2009) 244–249.
[128] R. Bucala, MIF, MIF alleles, and prospects for therapeutic intervention in autoimmunity, J. Clin. Immunol. 33 (1) (2013) 72–78.
[129] E.F. Morand, M. Leech, Macrophage migration inhibitory factor in rheumatoid arthritis, Front. Biosci. 10 (2005) 12–22.
[130] H.-R. Kim, et al., Macrophage migration inhibitory factor upregulates angiogenic factors and correlates with clinical measures in rheumatoid arthritis, J. Rheumatol. 34 (5) (2007) 927–936.
[131] C. Herder, et al., Association of systemic concentrations of macrophage migration inhibitory factor with impaired glucose tolerance and type 2 diabetes, Diabetes Care 29 (2) (2006) 368–371.
[132] H.-R. Kim, et al., Elevated levels of macrophage migration inhibitory factor in women with metabolic syndrome, Horm. Metab. Res. 43 (09) (2011) 642–645.
[133] Y.I. Sanchez-Zamora, et al., Altered macrophage and dendritic cell response in Mif-/- mice reveals a role of Mif for inflammatory-Th1 response in type 1 diabetes, J. Diabetes Res. 2016 (2016) 7053963.
[134] A. Foote, et al., Macrophage migration inhibitory factor in systemic lupus erythematosus, J. Rheumatol. 31 (2) (2004) 268–273.
[135] M.S. Shin, et al., Macrophage migration inhibitory factor serves as an upstream regulator of NLRP3 expression and subsequent IL-1beta production in human monocytes in response to lupus U1-snRNP immune complex, Am. Assoc. Immnol. (2017).
[136] L. Leng, et al., A small-molecule macrophage migration inhibitory factor antagonist protects against glomerulonephritis in lupus-prone NZB/NZW F1 and MRL./lpr mice, J. Immunol. 186 (1) (2011) 527–538.
[137] M. Niino, et al., Macrophage migration inhibitory factor in the cerebrospinal fluid of patients with conventional and optic-spinal forms of multiple sclerosis and neuro-Behcet's disease, J. Neurol. Sci. 179 (1–2) (2000) 127–131.
[138] C.J. Edwards, et al., Molecular profile of peripheral blood mononuclear cells from patients with rheumatoid arthritis, Mol. Med. 13 (1) (2007) 40.
[139] G. Benedek, et al., MIF and D-DT are potential disease severity modifiers in male MS subjects, Proc. Natl. Acad. Sci. 114 (40) (2017) E8421–E8429.
[140] L. Rijvers, et al., The macrophage migration inhibitory factor pathway in human B cells is tightly controlled and dysregulated in multiple sclerosis, Eur. J. Immunol. 48 (11) (2018) 1861–1871.
[141] H. Korf, et al., MIF inhibition interferes with the inflammatory and T cell-stimulatory capacity of NOD macrophages and delays autoimmune diabetes onset, PLoS ONE 12 (11) (2017) e0187455.
[142] M. Rodriguez-Sosa, et al., Proinflammatory cytokine MIF plays a role in the pathogenesis of type-2 diabetes mellitus, but does not affect hepatic mitochondrial function, Cytokine 99 (2017) 214–224.
[143] J.A. Ralph, et al., Identification of NURR1 as a mediator of MIF signaling during chronic arthritis: effects on glucocorticoid-induced MKP1, Am. J. Pathol. 177 (5) (2010) 2366–2378.
[144] R. Donn, et al., A novel 5′-flanking region polymorphism of macrophage migration inhibitory factor is associated with systemic-onset juvenile idiopathic arthritis, Arthritis Rheum. 44 (8) (2001) 1782–1785.
[145] J. Baugh, et al., A functional promoter polymorphism in the macrophage migration inhibitory factor (MIF) gene associated with disease severity in rheumatoid arthritis, Genes Immun. 3 (3) (2002) 170–176.
[146] I. Kang, R. Bucala, The immunobiology of MIF: function, genetics and prospects for precision medicine, Nat. Rev. Rheumatol. 15 (7) (2019) 427–437.
[147] B.J. Plant, et al., Cystic fibrosis, disease severity, and a macrophage migration inhibitory factor polymorphism, Am. J. Respir. Crit. Care Med. 172 (11) (2005) 1412–1415.
[148] O. Illescas, et al., Macrophage Migration Inhibitory Factor-173 G/C Polymorphism: A Global Meta-Analysis across the Disease Spectrum, Front. Genet. 9 (2018) 55.
[149] D.N. Assis, et al., A macrophage migration inhibitory factor polymorphism is associated with autoimmune hepatitis severity in US and Japanese patients, Dig. Dis. Sci. 61 (12) (2016) 3506–3512.
[150] Z. Han, et al., Genetic variant rs755622 regulates expression of the multiple sclerosis severity modifier D-dopachrome tautomerase in a sex-specific way, BioMed Res. Int. (2018).
[151] E.H. Doherty, P.V. Tilstam, R. Bucala, MIF and MIF2 have distinct but synergistic roles in CIA pathogenesis, Am. Assoc. Immnol. (2019).
[152] K. Meyer-Siegler, P.B. Hudson, Enhanced expression of macrophage migration inhibitory factor in prostatic adenocarcinoma metastases, Urology 48 (3) (1996) 448–452.
[153] B. Sun, et al., Macrophage migration inhibitory factor promotes tumor invasion and metastasis via the Rho-dependent pathway, Clin. Cancer Res. 11 (3) (2005) 1050–1058.
[154] J. Chesney, et al., An essential role for macrophage migration inhibitory factor (MIF) in angiogenesis and the growth of a murine lymphoma, Mol. Med. 5 (3) (1999) 181–191.
[155] E.J. Miller, et al., Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic hea
[156] S.E. Brock, et al., Negative regulation of A activity by macrophage migration inhibito small cell lung carcinomas, J. Biol. Chem. 287 (45) (2012) 37917–37925.
[157] D. Guo, et al., D-dopachrome tautomerase is over-expressed in pancreatic ductal adenocarcinoma and acts cooperatively with macrophage migration inhibitory factor to promote cancer growth, Int. J. Cancer 139 (9) (2016) 2056–2067.
[158] D. Xin, et al., The MIF homologue D-dopachrome tautomerase promotes COX-2 expression through β-catenin–dependent and–independent mechanisms, Mol. Cancer Res. 8 (12) (2010) 1601–1609.
[159] A.V. Sampey, et al., Regulation of synoviocyte phospholipase A2 and cyclooxygenase 2 by macrophage migration inhibitory factor, Arthrit. Rheumat.: Off. J. Am. Coll. Rheumatol. 44 (6) (2001) 1273–1280.

http://refhub.elsevie

[160] S. Choi, et al., Role of macrophage migration inhibitory factor in the regulatory T cell response of tumor-bearing mice, J. Immunol. 189 (8) (2012) 3905–3913.
[161] T. Pacheco-Fernández, et al., Macrophage migration inhibitory factor promotes the interaction between the tumor, macrophages, and T cells to regulate the progression of chemically induced colitis-associated colorectal cancer, Mediators Inflamm. 2019 (2019).
[162] K.L. Meyer-Siegler, et al., Inhibition of macrophage migration inhibitory factor or its receptor (CD74) attenuates growth and invasion of DU-145 prostate cancer cells, J. Immunol. 177 (12) (2006) 8730–8739.
[163] S. Ekmekcioglu, et al., Inflammatory marker testing identifies CD74 expression in melanoma tumor cells, and its expression associates with favorable survival for stage III melanoma, Clin. Cancer Res. 22 (12) (2016) 3016–3024.
[164] Z.-Q. Wang, et al., CD74 and intratumoral immune response in breast cancer, Oncotarget 8 (8) (2017) 12664.
[165] C. Otterstrom, et al., CD74: a new prognostic factor for patients with malignant pleural mesothelioma, Br. J. Cancer 110 (8) (2014) 2040–2046.
[166] K. Tanese, et al., Cell surface CD74–MIF interactions drive melanoma survival in response to interferon-γ, J, Invest. Dermatol. 135 (11) (2015) 2775–2784.
[167] W.Y. Weiser, et al., Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor, Proc. Natl. Acad. Sci. 86 (19) (1989) 7522–7526.
[168] C. Weber, et al., Structural determinants of MIF functions in CXCR2-mediated inflammatory and atherogenic leukocyte recruitment, Proc. Natl. Acad. Sci. 105 (42) (2008) 16278–16283.
[169] V. Veillat, et al., Involvement of nuclear factor-κB in macrophage migration inhibitory factor gene transcription up-regulation induced by interleukin-1β in ectopic endometrial cells, Fertil. Steril. 91 (5) (2009) 2148–2156.
[170] H. Lue, et al., Rapid and transient activation of the ERK MAPK signalling pathway by macrophage migration inhibitory factor (MIF) and dependence on JAB1/CSN5 and Src kinase activity, Cell. Signal. 18 (5) (2006) 688–703.
[171] H. Jung, et al., Regulation of macrophage migration inhibitory factor and thiol-specific antioxidant protein PAG by direct interaction, J. Biol. Chem. 276 (18) (2001) 15504–15510.
[172] M. Vujicic, et al., The critical role of macrophage migration inhibitory factor in insulin activity, Cytokine 69 (1) (2014) 39–46.
[173] L. Verschuren, et al., MIF deficiency reduces chronic inflammation in white adipose tissue and impairs the development of insulin resistance, glucose intolerance, and associated atherosclerotic disease, Circ. Res. 105 (1) (2009) 99–107.
[174] Z.-B. Shang, et al., Serum macrophage migration inhibitory factor as a biomarker of active pulmonary tuberculosis, Ann. Lab. Med. 38 (1) (2018) 9–16.
[175] S. Mahdian, et al., Macrophage migration inhibitory factor as a potential biomarker of endometriosis, Fertil. Steril. 103 (1) (2015) pp. 153–159.e3.
[176] A. Oikonomidi, et al., Macrophage migration inhibitory factor is associated with biomarkers of Alzheimer's disease pathology and predicts cognitive decline in mild cognitive impairment and mild dementia, J. Alzheimers Dis. 60 (1) (2017) 273–281.
[177] J. Wang, et al., Urinary macrophage migration inhibitory factor as a noninvasive biomarker in pediatric henoch-schönlein purpura nephritis, JCR J. Clin. Rheumatol. 23 (5) (2017) 258–261.
[178] R. Zwiech, Macrophage migration inhibitory factor urinary excretion revisited–MIF a potent predictor of the immunosuppressive treatment outcomes in patients with proliferative primary glomerulonephritis, BMC Immunol. 16 (1) (2015) 47.
[179] H. Otukesh, et al., Urine macrophage migration inhibitory factor (MIF) in children with urinary tract infection: a possible predictor of acute pyelonephritis, Pediatric Nephrol. 24 (1) (2009) 105.
[180] P.L. Vera, et al., Elevated urine levels of macrophage migration inhibitory factor in inflammatory bladder conditions: a potential biomarker for a subgroup of interstitial cystitis/bladder pain syndrome patients, Urology 116 (2018) 55–62.
[181] H. Zhang, et al., CXCL2/MIF-CXCR2 signaling promotes the recruitment of myeloid-derived suppressor cells and is correlated with prognosis in bladder cancer, Oncogene 36 (15) (2017) 2095.
[182] A.M. Patterson, et al., Human leukocyte antigen-presented macrophage migration inhibitory factor is a surface biomarker and potential therapeutic target for ovarian cancer, Mol. Cancer Ther. 15 (2) (2016) 313–322.
[183] S.-S. Wang, et al., Macrophage migration inhibitory factor: a potential driver and biomarker for head and neck squamous cell carcinoma, Oncotarget 8 (6) (2017) 10650.
[184] H.H.X. Xia, et al., Serum macrophage migration-inhibitory factor as a diagnostic and prognostic biomarker for gastric cancer, Cancer 115 (23) (2009) 5441–5449.
[185] H. Lee, et al., Macrophage migration inhibitory factor may be used as an early diagnostic marker in colorectal carcinomas, Am. J. Clin. Pathol. 129 (5) (2008) 772–779.
[186] B.-S. Kim, et al., The clinical significance of the MIF homolog d-dopachrome tautomerase (MIF-2) and its circulating receptor (sCD74) in burn, Burns 42 (6) (2016) 1265–1276.
[187] T.-Y. Chuang, et al., High levels of serum macrophage migration inhibitory factor and interleukin 10 are associated with a rapidly fatal outcome in patients with severe sepsis, Int. J. Infect. Dis. 20 (2014) 13–17.
[188] D. Qi, et al., The vestigial enzyme D-dopachrome tautomerase protects the heart against ischemic injury, J. Clin. Investig. 124 (8) (2014) 3540–3550.
[189] J. Pohl, et al., Myocardial expression of macrophage migration inhibitory factor in patients with heart failure, J. Clin. Med. 6 (10) (2017) 95.
[190] J. Pohl, et al., MIF reflects tissue damage rather than inflammation in post-cardiac arrest syndrome in a real life cohort, Resuscitation 100 (2016) 32–37.
[191] K. Koga, et al., Macrophage migration inhibitory factor provides cardioprotection during ischemia/reperfusion by reducing oxidative stress, Antioxid. Redox Signal. 14 (7) (2011) 1191–1202.
[192] Y. Ma, et al., Cardiomyocyte d-dopachrome tautomerase protects against heart failure, JCI Insight 4 (17) (2019).
[193] D. Qi, et al., Cardiac macrophage migration inhibitory factor inhibits JNK pathway activation and injury during ischemia/reperfusion, J. Clin. Investig. 119 (12) (2009) 3807–3816.
[194] C. Stoppe, et al., Interaction of MIF family proteins in myocardial ischemia/reperfusion damage and their influence on clinical outcome of cardiac surgery patients, Antioxid. Redox Signal. 23 (11) (2015) 865–879.
[195] X.-N. Deng, et al., Admission macrophage migration inhibitory factor predicts long-term prognosis in patients with ST-elevation myocardial infarction, Eur. Heart J.-Qual. Care Clin. Outcomes 4 (3) (2018) 208–219.
[196] F. Deng, et al., Prognostic significance and dynamic change of plasma macrophage migration inhibitory factor in patients with acute ST-elevation myocardial infarction, Medicine 97 (43) (2018).
[197] K. Nakahara, et al., Attenuation of macrophage migration inhibitory factor-stimulated signaling via S-nitrosylation, Biol. Pharm. Bull. 42 (6) (2019) 1044–1047.
[198] A. Schinagl, et al., Oxidized macrophage migration inhibitory factor is a potential new tissue marker and drug target in cancer, Oncotarget 7 (45) (2016) 73486.
[199] X. Chen, et al., Prevention of acute graft-versus-host disease in a xenogeneic SCID mouse model by the humanized anti-CD74 antagonistic antibody milatuzumab, Biol. Blood Marrow Transplant. 19 (1) (2013) 28–39.
[200] D. Frölich, et al., The anti-CD74 humanized monoclonal antibody, milatuzumab, which targets the invariant chain of MHC II complexes, alters B-cell proliferation, migration, and adhesion molecule expression, Arthritis Res. Therapy 14 (2) (2012) R54.
[201] J.L. Kaufman, et al., Phase I, multicentre, dose-escalation trial of monotherapy with milatuzumab (humanized anti-CD74 monoclonal antibody) in relapsed or refractory multiple myeloma, Br. J. Haematol. 163 (4) (2013) 478–486.
[202] B.A. Christian, et al., The combination of milatuzumab, a humanized anti-CD74 antibody, and veltuzumab, a humanized anti-CD20 antibody, demonstrates activity in patients with relapsed and refractory B-cell non-Hodgkin lymphoma, Br. J. Haematol. 169 (5) (2015) 701–710.
[203] M. Haran, et al., A phase I-II clinical trial of the anti-CD74 monoclonal antibody milatuzumab in frail patients with refractory chronic lymphocytic leukaemia: A patient based approach, Br. J. Haematol. 182 (1) (2018) 125.
[204] M.C. Zapatero, et al., Discovery of novel inhibitors of the tautomerase activity of macrophage migration inhibitory factor (MIF), J. Biomol. Screen. 21 (5) (2016) 446–458.
[205] H. Ouertatani-Sakouhi, et al., Identification and characterization of novel classes of macrophage migration inhibitory factor (MIF) inhibitors with distinct mechanisms of action, J. Biol. Chem. 285 (34) (2010) 26581–26598.
[206] Y. Cho, et al., Allosteric inhibition of macrophage migration inhibitory factor revealed by ibudilast, Proc. Natl. Acad. Sci. 107 (25) (2010) 11313–11318.
[207] J. Bloom, et al., Identification of iguratimod as an inhibitor of macrophage migration inhibitory factor (MIF) with steroid-sparing potential, J. Biol. Chem. 291 (51) (2016) 26502–26514.
[208] I. Saito, et al., Neuroprotective effect of an antioxidant, ebselen, in patients with delayed neurological deficits after aneurysmal subarachnoid hemorrhage, Neurosurgery 42 (2) (1998) 269–277.
[209] N. Ishiguro, et al., Investigation of the predictors of the response to Iguratimod therapy: A post-hoc analysis of post-marketing surveillance study, Mod. Rheumatol. (2019) 1–7.
[210] R.J. Fox, et al., Phase 2 trial of ibudilast in progressive multiple sclerosis, N. Engl. J. Med. 379 (9) (2018) 846–855.
[211] G. Pantouris, R. Bucala, E.J. Lolis, Structural plasticity in the C-terminal region of macrophage migration inhibitory factor-2 is associated with an induced fit mechanism for a selective inhibitor, Biochemistry 57 (26) (2018) 3599–3605.
[212] G. Fingerle-Rowson, et al., A tautomerase-null macrophage migration-inhibitory factor (MIF) gene knock-in mouse model reveals that protein interactions and not enzymatic activity mediate MIF-dependent growth regulation, Mol. Cell. Biol. 29 (7) (2009) 1922–1932.
[213] Y.-X. Zheng, et al., CD74 and macrophage migration inhibitory factor as therapeutic targets in gastric cancer, World J. Gastroenterol. 18 (18) (2012) 2253–2261.
[214] P. Fagone, et al., Contribution of the macrophage migration inhibitory factor superfamily of cytokines in the pathogenesis of preclinical and human multiple sclerosis: In silico and in vivo evidences, J. Neuroimmunol. 322 (2018) 46–56.
[215] M. Winner, et al., A novel, macrophage migration inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells, Cancer Res. 68 (18) (2008) 7253–7257.
[216] L. Varinelli, et al., 4-IPP, a selective MIF inhibitor, causes mitotic catastrophe in thyroid carcinomas, Endocr. Relat. Cancer 22 (5) (2015) 759–775.
[217] Y. Zheng, et al., Role of myeloma-derived MIF in myeloma cell adhesion to bone marrow and chemotherapy response, JNCI: J. Natl. Cancer Instit. 108 (11) (2016).
[218] K. Husain, et al., Protection by ebselen against cisplatin-induced nephrotoxicity: antioxidant system, Mol. Cell. Biochem. 178 (1–2) (1998) 127–133.
[219] P.D. Hanavan, et al., Ebselen inhibits QSOX1 enzymatic activity and suppresses invasion of pancreatic and renal cancer cell lines, Oncotarget 6 (21) (2015) 18418.
[220] J. Wang, et al., Limiting cardiac ischemic injury by pharmacological augmentation of macrophage migration inhibitory factor–AMP-activated protein kinase signal transduction, Circulation 128 (3) (2013) 225–236.

Articulo 5: Jonadab E. Olguín, Itzel Medina-Andrade, Tonathiu Rodríguez, Miriam Rodríguez-Sosa and Luis I. Terrazas. 2020. Relevance of Regulatory T Cells during Colorectal Cancer Development.Cancers. IF 6.126

cancers

MDPI

Review

Relevance of Regulatory T Cells during Colorectal Cancer Development

Jonadab E. Olguín [1,2], Itzel Medina-Andrade [1,2], Tonathiu Rodríguez [1], Miriam Rodríguez-Sosa [1] and Luis I. Terrazas [1,2,*]

1 Laboratorio Nacional en Salud, Diagnóstico Molecular y Efecto Ambiental en Enfermedades Crónico-Degenerativas, Facultad de Estudios Superiores (FES) Iztacala, Universidad Nacional Autónoma de México (UNAM), Av. De los Barrios # 1, Tlalnepantla 54090, Mexico; efra004@gmail.com (J.E.O.); itzelmedina.andrade@gmail.com (I.M.-A.); tonathiurh@hotmail.com (T.R.); rodriguezm@unam.mx (M.R.-S.)

2 Unidad de Biomedicina, FES Iztacala, UNAM, Av. De los Barrios # 1, Tlalnepantla 54090, Mexico

* Correspondence: literrazas@unam.mx; Tel.: +52-5556-239-794

Received: 27 May 2020; Accepted: 1 July 2020; Published: 14 July 2020

check for updates

Abstract: In recent years, there has been a significant increase in the study of own and foreign human factors favoring the development of different types of cancer, including genetic and environmental ones. However, the fact that the immune response plays a fundamental role in the development of immunity and susceptibility to colorectal cancer (CRC) is much stronger. Among the many cell populations of the immune system that participate in restricting or favoring CRC development, regulatory T cells (Treg) play a major role in orchestrating immunomodulation during CRC. In this review, we established concrete evidence supporting the fact that Treg cells have an important role in the promotion of tumor development during CRC, mediating an increasing suppressive capacity which controls the effector immune response, and generating protection for tumors. Furthermore, Treg cells go through a process called "phenotypic plasticity", where they co-express transcription factors that promote an inflammatory profile. We reunited evidence that describes the interaction between the different effector populations of the immune response and its modulation by Treg cells adapted to the tumor microenvironment, including the mechanisms used by Treg cells to suppress the protective immune response, as well as the different subpopulations of Treg cells participating in tumor progression, generating susceptibility during CRC development. Finally, we discussed whether Treg cells might or might not be a therapeutic target for an effective reduction in the morbidity and mortality caused by CRC.

Keywords: regulatory T cells; colorectal cancer; animal models; clinical trial; phenotypic plasticity

1. Introduction

Traditional treatments to reduce the mortality of cancer, such as surgery, radiotherapy, or chemotherapy, have shown limitations in their effectiveness. In recent years, emphasis has been placed on the importance of the immune response as an orchestrator of resistance for different types of cancers [1]. Immunotherapy is a way of reducing the mortality caused by cancer [2], for example, the adoptive transfer of antigen-specific T cells, which has been shown to generate a positive immune response in metastatic melanoma [3]. The use of monoclonal antibodies directed against specific molecular and cellular targets, as well as the use of immunotherapy based on T cell clones in gastric cancer, are now relatively affordable [2,4]. It has been shown that the use of NK cells activated with IL-2 during acute myeloid leukemia can generate complete remission in 26% of patients [5]. The use of anti-PD-1 and programmed cell death ligand 1 (PD-L1) antibodies have shown long-lasting responses in non-small cell lung cancer, with a favorable safety profile and manageable side effects [6].

During colorectal cancer (CRC), some immunotherapy clinical trials have demonstrated a potential benefit, but most of them remain as experimental options. Vaccines directed against overexpressed peptides in mucosal tissues of patients with CRC, which can induce tumor antigen-specific immune response with a lower risk of inducing autoimmunity, have been used [7]. Dendritic cell transfer pulsed with tumor epitopes or tumor cell lysates have been used to induce immunity to gastric cancer [7]. As mentioned above, some immunologic strategies have been used as treatment against some types of cancers, showing effectiveness, but continuous research development is necessary for a clear panoramic understanding of the role of the immune response during CRC.

2. Regulatory T Cells: Brief Summary

Since regulatory T (Treg) cells were described in 1995 by Shimon Sakaguchi, they have been protagonists in most immunological processes caused by self or non-self antigens in parasitic, autoimmune, inflammatory, and oncological diseases [8]. First, Treg cells were described as a subpopulation of T cells that suppressed the immune response, avoiding autoimmunity, and were characterized by the expression of the alpha chain receptor for IL-2 [9], also called CD25, which had previously been found expressed on activated T cells [10,11]. After a long race to determine if a specific marker for Treg cells exists, it was shown that the forkhead box p3 transcription factor (Foxp3) is the molecule conferring suppressor activity on Treg cells [12,13]; thus, Foxp3 is the master regulator of Treg cells [14]. Mice and humans with a mutation in the *Foxp3* gene display a T cell-dependent, lymphoproliferative immune disorder manifested by some diseases, such as type-1 diabetes, thyroiditis, splenomegaly, and lymphadenopathy [15].

Treg cells use several mechanisms to suppress immune responses, such as deprivation of IL-2 by its IL-2 (CD25) high-affinity receptor (Figure 1A) [16–19], the use of CD39 and CD73 ectoenzymes for the release of extracellular adenosine (Figure 1A), which is a strong immunosuppressant [20–22], the secretion of suppressor cytokines such as IL-10 [23], TGF-β [24,25] and IL-35 [26,27] (Figure 1B), the manipulation of antigen-presenting cells by inducing a "tolerant phenotype" through Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), and the Lymphocyte Activation Gene-3 (LAG-3) to induce the Indoleamine 2,3-dioxygenase (IDO) enzyme, which in turn reduces the availability of tryptophan in the environment along the kynurenine pathway (Figure 1C) [28–30]. In humans, it has also been reported that Treg cells use granzyme and perforin-like molecules as a suppressive mechanism (Figure 1D) [31,32].

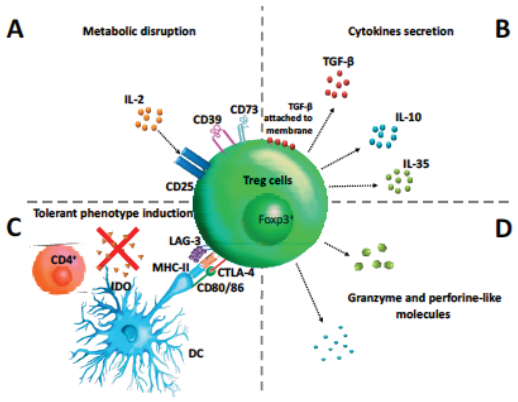


Figure 1. Natural regulatory T (Treg) cells and their main suppressive mechanisms. (**A**) Metabolic disruption of IL-2 caused by an increased expression of CD25 (high-affinity IL-2 receptor) in Treg cells, also caused by the release of extracellular adenosine. (**B**) Secretion of cytokines such as IL-10, TGF-β, and IL-35. (**C**) Manipulation of antigens presenting cells for a tolerant phenotype. (**D**) Secretion of granzyme and perforin.

Besides the expression of CD25 and the Foxp3 transcription factor, Treg cells also display some molecules associated with activation in their surface, which confer on them a higher suppressive capacity, such as Glucocorticoid-Induced Tumor Necrosis Factor receptor (GITR), Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4), Inducible T-cell Costimulator (ICOS) [33], Programmed cell Death protein 1 (PD-1) [34], and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) [35] (Figure 2A). All these features make Treg cells a versatile immune population with a wide range of mechanisms that could be manipulated either for or against the protection of health.

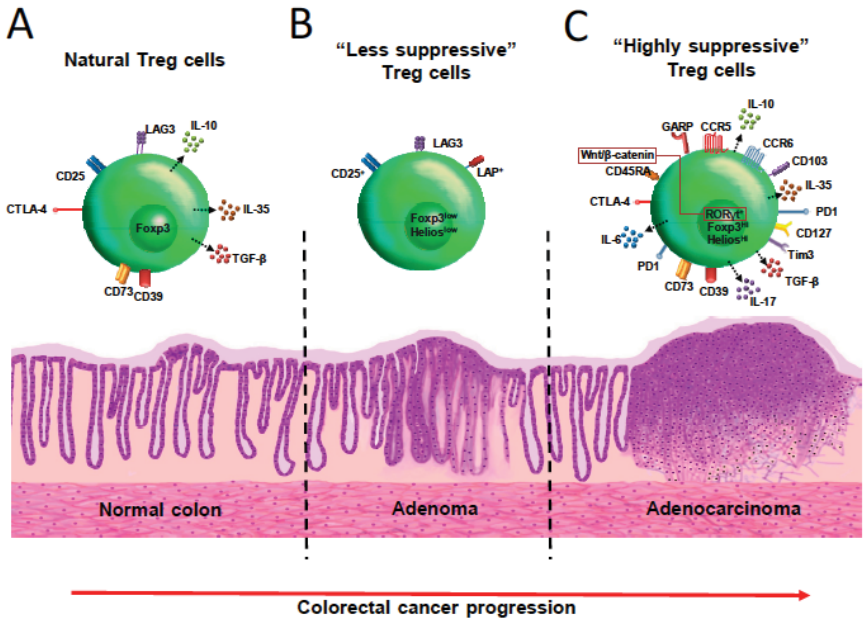


Figure 2. Phenotype of Treg cells in the progression of CRC. As mentioned in the text, adenomas are the precursors of CRC, arising from the adenoma-carcinoma sequence. (**A**) When the intestinal tissue has a normal condition, natural Treg cells display a regular phenotype, but the genetic, epigenetic, and mainly the immunological alterations that end in the formation of adenomas, modify the phenotype in Treg cells, which confers different roles, depending of the grade of alterations during CRC. We included these subpopulations of Treg cells in 2 groups: (**B**) "less suppressive" Treg cells which are associated with an immunological protection against tumor formation, and (**C**) "Highly suppressive" Treg cells, whose phenotype is associated with tumor progression and a poor protective immune response against CRC.

3. Treg Cells during CRC in Clinical Cases: An Overview

Colorectal cancer is one of the most common and fatal cancers in the world [36], being the third most common cancer worldwide, and the second most deadly, just behind lung cancer [37]. The incidence rates are higher in developed countries, but the mortality rate is much higher in developing ones [38–40]. CRC has different origins: hereditary, sporadic, and colitis-associated colon cancer (CAC). Only 5% of CRC cases are hereditary, whereas 75% are sporadic, being associated with environmental factors, and the remaining 20% are associated with dysregulated inflammatory responses in the colon [41]. An aggressive and prolonged inflammation generates adenomas in colon tissues [42]. Colorectal adenomas are lesions with dysplastic epithelium, characterized by being benign in general, and dysplasia that could be either low or high is mainly determined by abnormal nuclear morphology [43]. However, adenomas are the precursors of CRC, arising from the adenoma-carcinoma

sequence; the risk of malignancy increases along with polyp size and degree of dysplasia [43]. Both genetic and epigenetic alterations contribute to the formation of immunogenic tumor-specific and tumor-associated antigens [44], which allow the identification and elimination of CRC by the immune response [45,46]. However, some cells of the immune response play a role in the initial inflammation, resulting from tumor initiation up to metastasis during CRC. For example, myeloid cells, such as monocytes, macrophages, and neutrophils, secrete cytokines and express immunomodulatory molecules in their surface, which may promote the development of the tumor and avoid its elimination, through the induction and maintenance of an immunosuppressive microenvironment [47,48].

Similarly, aiming to induce immunosuppression, Treg cells have been associated with tumor progression during CRC (Figure 2) [49]. Interestingly, CRC patients display Treg cells with a higher expression of several molecules that correlate with suppression, such as Tim-3, LAG-3, TGF-β, IL-10, CD25, and CTLA-4 [50], and by the BLIMP-1 transcription factor expression in the tumor [51]. The infiltration of Treg cells into the colon is significantly higher in CRC than in a healthy colon, as well as in patients with limited disease (Union for International Cancer Control (UICC) criteria I and II) than in metastatic (UICC criteria III and IV) ones [52]. This was also associated with the frequency of $Foxp3^+$ cells in patients with lymphatic invasion [53]. The increase in Foxp3 expression in colorectal tissues and peripheral blood correlates with an increasing degree of tumor malignancy and lymph-node metastasis [54]. It was also demonstrated that patients with CRC had increasing percentages of Treg cells in the peripheral blood and mesenteric lymph nodes compared to either healthy controls or patients with inflammatory bowel disease. Additionally, in this study, it was observed that when Treg cells from CRC patients were depleted in peripheral blood, $CD4^+$ cells produced IFN-γ in a specific-antigen shape against the tumor antigen 5T4 but not in the control samples [55]. Therefore, Treg cells can inhibit an anti-tumor specific immune response in patients with CRC. Another study showed that the density of Treg cells was dramatically higher in tumor-draining lymph nodes than in peripheral blood or tumor-infiltrating lymphocytes, and these data were correlated with the staging of the disease. Furthermore, the $CD8^+$ T cell function was restored after Treg depletion [56]. More recently, it was found that patients with colon adenocarcinoma displayed an accumulation of Treg cells overexpressing PD-1, which impaired $CD8^+$ T cells activity in situ [57]. Treg cells in tumor patients are specific for a limited repertoire of tumor antigens, suggesting that these cells exert strong T cell suppression in an antigen-selective manner during CRC, and the effector/memory T cell response against antigens recognized by Treg cells strongly increases after Treg cell depletion [58]. Thus, the evidence for a role of Treg cells as part of the immunosuppressive microenvironment that promotes the development of tumors during CRC has been described, and it suggests that the frequency of sub-populations of Treg cells may provide a useful tool with possible prognostic value for the treatment of CRC.

4. Treg Cells during CRC in Murine Models

The use of animal models is a tool that grants easier access to samples, for the quantity of samples and for the short period of time it takes to obtain results. Many ideas applied in the clinic came from experiments designed and performed in murine models. With this in mind, the study of Treg cells has been applied to CRC in murine models. One model of CRC in mice was described by Tanaka in 2003, where azoxymethane (AOM) and dextran-sodium sulfate (DSS) were used. Mice were injected with AOM (which exerts colonotropic carcinogenicity), 12.5 mg/kg; then, 7, 29, and 51 days after AOM injection, 2% DSS (for colitis induction) was added to the drinking water for 7 days. This model is called CAC and it displays many similarities to CRC in humans, but with the advantage of obtaining results faster. It has been demonstrated that at the final stage of CAC development [59], Treg cells increase in number and exhibit a phenotype of activation defined by the expression of CD103, Receptor Glycoprotein-A Repetitions Predominant (GARP), CTLA-4, and IL-10 (Figure 2C) [60]. This is in accordance with the idea that a correlation between Foxp3 expression and tumor progression during CRC exists [61]. The transient ablation of Treg cells using depletion of Treg cells (DEREG) mice, which express the diphtheria toxin receptor under the Foxp3 promotor [62], suppressed colon tumor

size; however, the mortality rate for these mice increased [60]. In our lab, we were interested in the dynamic behavior of Treg cells during the development of CAC, focusing not only on the final phase of tumor formation but also on all the phases of its development. Using transgenic Foxp3^{EGFP} mice and the CAC model, we observed a reduced percentage of Treg cells in blood and spleen during early CAC development, and an increased percentage of these cells was shown at late stages of CAC in mesenteric lymph nodes (MLN) [63]; these observations have been confirmed in patients with CRC [64]. Conversely, at early stages of CAC, a higher percentage of activated T cells (Tact) were observed, but as CAC progressed it was detected that Tact cells were significantly reduced. Interestingly, Treg cells from late stages of CAC displayed an activated phenotype featured by increased expression of PD-1, Tim-3, and CD127 molecules in their membranes (Figure 2C). Moreover, these Treg cells from CAC mice obtained from MLN suppressed CD4$^+$ and CD8$^+$ T-activated cells in a more efficient way than healthy wild-type mice. Thus, with the idea of inhibiting the accumulation of Treg cells during CAC development, we used the PC61 monoclonal antibody (anti-CD25) during the early phase of CAC development to reduce the percentage of Treg cells; this early intervention guarantees the reduction mainly in Treg and not in Tact cell population [63]. Reduction of 50% of Treg cells resulted in a better prognostic value by a significant reduction in the tumor load, which was associated with an increased percentage of both CD4$^+$ and CD8$^+$ T-activated cells in MLN in CAC mice receiving immunotherapy with the monoclonal antibody PC61. All these results suggest that Treg cells play a critical role by suppressing the immune response in the early stages of CAC development [63].

The inoculation of colorectal carcinoma tumor cells has also been used for the study of Treg cells during CRC development in mouse models. In this orthotopic mouse model, it has been reported that Treg cell depletion using PC61 antibody before the inoculation of colorectal carcinoma tumor cells CT26 resulted in protective immunity mediated by CD8$^+$ cytotoxic T cells; thus, the specificity of cytotoxic responses to tumor antigens can be suppressed by Treg cells [65]. As mentioned above, Treg cells express the ecto-enzyme CD73, which together with CD39 can hydrolyze the extracellular ATP in adenosine, a strong immunosuppressant. It is known that extracellular adenosine is accumulated in the tumor microenvironment, suppressing the anti-tumor immune response (Figure 2C). Adenosine levels can increase in response to chronic inflammation, which is a characteristic of CRC. Thus, CD73 ablation significantly suppressed the growth of the MC38 colon cancer cell line, in a CD8$^+$ T cell-dependent pathway. This effect was associated with an increased level of both antigen-specific CD8$^+$ T cells and IFN-γ production in peripheral blood and locally in the tumors [66].

In the *adenomatous polyposis coli* (APC) animal model of human familial adenomatous polyposis (FAP), mice develop numerous polyps in the intestinal tract due to a truncation in the APC gene [67,68]. This model has been used as a tool for the evaluation of anticancer, chemo-preventive agents, and also for the study of the immune response against CRC [67,68]. During the CRC development in the APC$^{min/+}$ mouse model, CD4$^+$Foxp3$^+$ Treg cells accumulate in the adenomas, which match with lower frequencies of conventional T and B cells in situ [69], indicating a downmodulation of the local immune response against CRC. Furthermore, in this model, adenomas displayed an altered chemokine profile with high levels of CCL17 and low levels of CXCL11 and CCL25, and their Treg cells did not express CXCR3 [69]. By breeding APC$^{min/+}$ mice with DEREG mice (see above), it was possible to selectively deplete Treg cells in tumor-bearing mice. This exclusive depletion of Treg cells increased the frequency, infiltration, and proliferation of T cells in the tumors, which correlated with increased expression of CXCR3$^+$ T cells and IFN-γ production [70,71]. CXCR3 and its ligands are differentially expressed at sites of inflammation and within the CRC tumors; CXCR3 is functionally expressed on Treg cells and also induces the differentiation of peripheral T cells into Treg cells, suggesting that a CXCR3 molecule could be an indirect Treg cell target with therapeutic potential during CRC [72]. In an APC$^{min/+}$ murine model, it was demonstrated that the oral administration of IL-10 encapsulated in microparticles reduces polyposis and increases the survival rate, apparently this controversial IL-10 effect can be explained given that IL-10 has a neutralization effect over Foxp3$^+$RORγt$^+$IL-17$^+$ Treg cells that promote the disease and a positive effect on the restauration of Foxp3$^+$RORγt$^-$IL-17$^-$ Treg cells,

which are protective (Figure 2C) [73]. Recently, in the APC$^{min/+}$/DEREG mouse model, it was shown that Treg cells specifically suppressed the TCRαβ^{+} CD8^{+} T cell population in colon tumors; when Treg cells were depleted, an increased amount of granzyme B and IFN-γ was observed in CD8^{+} T cells [74]. In another study, it was demonstrated that the adoptive transfer of T cells secreting IL-10 attenuated microbial-induced inflammation, suppressing polyposis in APC$^{\Delta 458}$ mice. In contrast, the ablation of IL-10 specifically in T cells produced pathologies like in systemic IL-10 deficient mice, increasing the number and growth of colon polyps. Treg cells and T cells are the major source of IL-10 in healthy colons and in colons containing polyps in this model [75]. Additionally, mice receiving broad-spectrum antibiotics presented a reduction in the microbiota, inflammation, and polyposis, suggesting that polyposis is fueled by a high number of microbes that accumulate in the colon, which in turn activate the inflammatory response; this inflammation is suppressed by IL-10 secreted by T and Treg cells [75]. It is clear that Treg cells play a role in the promotion of tumor development and in the reduction in an efficient immune response, but the results described above, where IL-10 from Treg cells play a role in the reduction in colon damage [73,75], are remarkably contrasting. These controversial findings could probably be explained by the fact that many subpopulations of Treg cells secreting different kinds of cytokines have different roles during CRC in both clinical and mouse models. However, these models highlight the importance for the microbiota in the context of CRC and Treg cells, because the higher densities of microbes that accumulate within polyps trigger local inflammatory responses, which are suppressed by IL-10 derived from both T and Treg cells. All these results suggest a close relationship between Treg cells and microbiota promoting tumor development during CRC.

5. Subpopulations of Treg Cells during CRC

In the past, evidence was collected suggesting that once a CD4^{+} T-lymphocyte acquired T_H1 (mainly secreting IFN-γ and inflammatory cytokines) or T_H2 (mainly secreting IL-4, and inhibitory T_H1 cytokines, promoting the antibody secretion) phenotypes, it was permanent during the functional life-span of the CD4^{+} T cell [76]. However, some years ago evidence emerged supporting the idea that CD4^{+} T cells actually can change their phenotype and function, being more flexible than expected in the production of cytokines [77]. This is because transcription factors such as T-bet, GATA-3, RORγt, and Foxp3 are expressed transiently, or because the CD4^{+} T-cells can express more than one transcription factor at the same time [78]. This kind of phenotypic plasticity was not only described in CD4^{+} T cells but also proposed in myeloid cells, including macrophages, mast cells, and neutrophils [79]. Treg cell phenotypic plasticity has also been suggested during CRC development, where an increased number of Treg cells Foxp3^{+} expressing IL-17 during human CRC was shown. These Treg cells also expressed CCR6^{+} TGF-β^{+} and IL-6^{+} (Figure 2C). This population, called Foxp3^{+}IL-17^{+} Treg cells, was more suppressive against CD8^{+} T-activated cells, and surprisingly, this suppression was reversed in the presence of an anti-IL-17 blocking antibody [80]. Foxp3^{+}IL-17^{+} Treg cells are selectively accumulated in the colonic microenvironment associated with colon carcinoma, and these types of Treg cells also favor inflammatory cytokine production in colon tissues. These data suggest that Foxp3^{+}IL-17^{+} Treg cells probably facilitate a chronic inflammatory pathological microenvironment in the colon, thus promoting tumor development [81]. In line with this evidence, a preferential expansion of a Foxp3^{+}RORγt^{+} subpopulation of Treg cells emerges in human CRC. These cells show a potent suppressive capacity but with an anti-inflammatory compromised ability (Figure 2C). These Foxp3^{+}RORγt^{+} Treg cell populations with the same suppressor abilities were shown in both a mouse model of hereditary polyposis [82] and an AOM/DSS CAC model [83]. The specific ablation of RORγt gene in Foxp3^{+} T cells improved the polyp-specific immune surveillance and attenuated the polyposis, indicating the inflammatory activity of these cells. Interestingly, the ablation of IL-6, IL-23, or IL-17 reduced the number of polyps but not in the same way that ablation of the RORγt gene did [84]. It was demonstrated that Wnt/β-catenin signaling in T cells promotes the expression of RORγt, which in turn promotes Th17-mediated inflammation (Figure 2C). In addition, the expression of β-catenin is increased in Treg cells from both mice and patients with CRC. The activation of β-catenin only in Treg

cells was enough to generate inflammation and carcinogenesis [85]. This evidence supports the idea that a subpopulation of Treg cells with a T_H17-like profile may exert both powerful inflammatory damage and strong immunosuppression during CRC development.

We previously mentioned that some studies on Treg cells are controversial, because the presence of Treg cells and their suppression mechanisms are involved in either a better or worse prognosis during CRC [73,75,86]. Due to this evidence, we think that some subpopulations of Treg cells likely have different roles at the same time. In fact, it has been demonstrated that during CRC development, Treg cells could be classified into two subpopulations by the grade of Foxp3 expression in $Foxp3^{lo}$ or $Foxp3^{hi}$. The $Foxp3^{lo}$ Treg cells are not suppressive (Figure 2B), and do not express the CD45RA receptor but secrete inflammatory cytokines. CRC patients with abundant $Foxp3^{lo}$ Treg cells showed a significantly better prognosis [87]. In another study, it was demonstrated that most of the intra-tumor $CD4^+Foxp3^+$ Treg cells have a $Helios^+$, CTLA-4^+, and $CD39^+$ phenotype, but 30% of $CD4^+Foxp3^-$ cells also expressed markers associated with regulatory functions, including CD25, LAG-3, and latency-associated peptide (LAP) (Figure 2B). This adaptive Treg subpopulation also produced IL-10 and TGF-β, and it was 50 times more suppressive than $CD4^+Foxp3^+$ Treg cells [88]. Helios is a member of the Ikaros transcription factor family and is preferentially expressed in Treg cells. It has been suggested that Helios is a marker for thymus natural Treg cells and not for induced Treg cells [89]. $Helios^{low}Foxp3^+$ Treg cells are enriched both in peripheral blood and at the tumor site (Figure 2B), but only $Helios^{hi}Foxp3^+$ Treg cells accumulate significantly and specifically in tumors (Figure 2C) and produce IL-17 during CRC. $CD4^+Foxp3^+Helios^+$ Treg cells co-expressed the suppressive molecules PD-1, CTLA-4, and CD39, suggesting that the phenotype of these Treg cells in the tumor of CRC patients is highly suppressive (Figure 2C) [90]. The manipulation of subpopulations of Treg cells, such as $Foxp3^{lo}$/$Foxp3^{hi}$ Treg cells co-expressing Helios, LAP, or another molecule associated with immunosuppression, could be useful targets to develop a strategy for an effective fight against CRC.

Another molecule directly associated with the suppressive activity of Treg cells is the IL-35 cytokine, which confers regulatory activity on naïve $CD4^+$ T cells and also suppresses T-cell proliferation [26,27]. Interestingly, IL-35 levels have been found to be elevated in both serum and tumors in patients with CRC, and they were correlated with tumor metastasis. Moreover, Treg cells from CRC patients were also capable of secreting high levels of IL-35 (Figure 2C) [91].

The chemokine receptor CCR5 has been involved in the recruitment of systemic Treg cells during CRC, and $CCR5^{-/-}$ mice have a delayed tumor growth because a reduced number of Treg cells are infiltrated in the tumors [92]. In human CRC, functional CCR5 was highly expressed in tumor-infiltrating Treg cells, and Treg cells expressing high levels of CCR5 are more suppressive; however, the pharmacological inhibition of CCR5 failed to reduce the tumor-infiltrating Treg cells, suggesting that other chemokine receptors are probably involved in the recruitment of Treg cells into the tumor during CRC development [93]. Perhaps a combined immunotherapy seeking to block two or more targets may favor a better prognosis against CRC in the clinic, but the establishment of more feasible therapeutic targets to improve the immunomodulation in CRC is clearly necessary.

6. Immune Molecules and Cells Promoting or Inhibiting Treg Cell Activity during CRC

We have described above the different suppression modes of Treg cells during CRC and how Treg cells are classified into subpopulations with specialized immunosuppressant features (Figure 2). Next, we are going to establish that Treg cells and several molecules and immune cells are involved in the promotion or inhibition of Treg cells during CRC in animal models and in clinical trials.

6.1. TGF-β1, Runt-Related Transcription Factor (RUNX) 3, and GARP

TGF-β1 is essential for the maintenance of inflammatory homeostasis; loss of its signaling is involved in malignant tumor formation [94]. RUNX is a family of proteins that participate down-stream of TGF-β1 signaling, and their loss is involved in severe inflammation and tumor formation in the gastrointestinal tract [95]; RUNX3 is involved in the differentiation of CD8 and NK cells; thus, $Runx3^{-/-}$

mice develop immunodeficiency by the absence of these cells [96]. Using stylish experiments with chimeric mice, it has been demonstrated that the loss of RUNX3, specifically in T cells, resulted in an impaired suppressive ability of the Tregs, as well as a reduction in the numbers of induced Treg cells by TGF-β stimulation (Figure 3A). This lack of activity of Treg cells caused colitis and the development of tumors in the large intestine and cecum, when animals were housed in a conventional animal facility. In the same way, no tumor was detected when $CD8^+$ T or Treg cells from WT origin were transferred into chimeric mice; however, the tumor formation was completely blocked by housing animals in a pathogen-free condition, suggesting that microbiota is involved in tumor development [97]. These results are controversial with data where Treg cells are involved in the promotion of tumor growth and reduced immune response, which leads to the development of CRC. Probably these data represent an example of a subpopulation of Treg cells acting against tumor formation. The GARP molecule expressed on the surface of Treg cells is involved in the activation of TGF-β signaling; thus, the specific absence of GARP in Treg cells in a CAC model improved anti-tumor immunity [98].

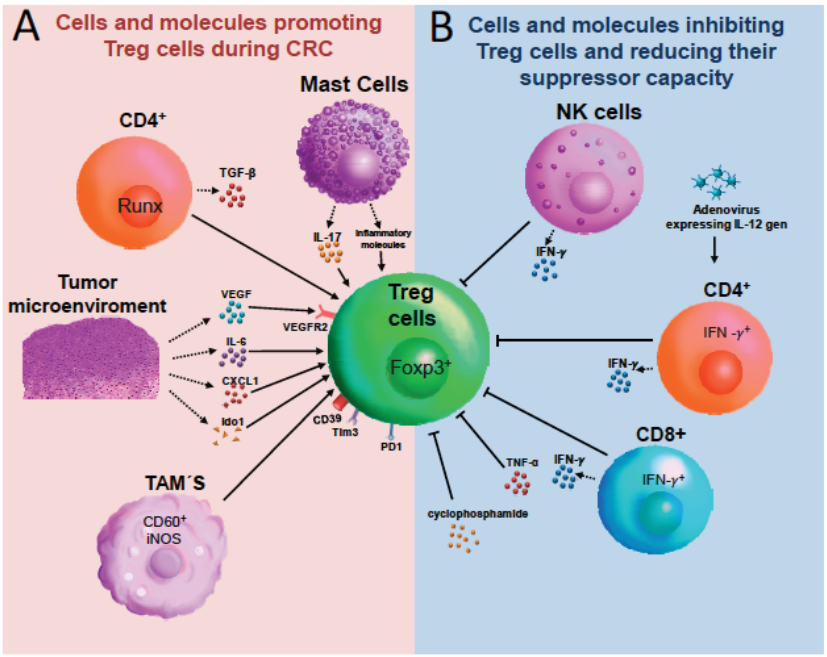


Figure 3. Different molecules and cells either promoting or inhibiting Treg cell activities during CRC. (**A**) As described in the text, some cells, such as mast cells, T cells, and TAMs, are involved in the induction of Treg cell population during CRC development. Additionally, the same tumor microenvironment produces molecules to induce a more suppressive population of Treg cells. (**B**) On the other hand, cells such as NK, CD4, and CD8 T cells, are involved in the inhibition of the suppressor capacity of Treg cells during CRC.

6.2. IL-33 Receptor

Another interesting molecule is the IL-33 receptor (ST2 or IL1RL1), which is involved in the stimulation of the suppressive functions of Treg cells in physiological and pathological conditions [99]. ST2 is highly expressed in tumors of CRC patients, which correlates with an increased expression of Foxp3 in both adenoma and CRC tissues. A higher density of ST2 expression in tumor samples is associated with increased dysplasia [100].

6.3. Vascular Endothelial Growth Factor (VEGF)

A molecule involved in Treg cell promotion is VEGF, which is both secreted and abundant in the tumor microenvironment and suppresses anti-tumor immunity. Its receptor, VEGFR2, is expressed selectively in intra-tumor Treg cells with high expression of Foxp3 (Figure 3A) [101], and it is proposed that both VEGFR2 and Foxp3 may be better predictive markers for recurrence and survival in patients with CRC [101].

6.4. Indoleamine 2,3-Dioxygenase (IDO)

In different types of tumors, the IDO enzyme expression has been found to be increased in tumor tissue and in draining lymph nodes, and it is believed that it plays a role in tumor evasion by suppressing the immune response [28]. Ido1$^{-/-}$ mice (Ido1 is a paralog enzyme involved in the degradation of tryptophan) either bred with APC$^{min/+}$ mice or in the CAC model did not lead to significant differences in the size and number of colon tumors. However, Ido1 deficiency altered the immune response in the tumor microenvironment with increased levels of pro-inflammatory cytokines and a reduced number of Treg cells (Figure 3A). Thus, the exclusive elimination of IDO is not sufficient to reduce the progression of colon cancer [102].

6.5. CD39

As previously mentioned, CD39 is an ecto-enzyme that mediates the generation of immunosuppressive adenosine [20,21]. Treg cells from CRC patients express high levels of CD39 [103], which may increase the amount of adenosine available in the tumor microenvironment. Adenosine reduces the capacity of monocytes to activate the endothelium, which indirectly affects T cells' recruitment to reach the site of the tumor. Thus, Treg-derived adenosine acts on monocytes and not only helps reduce trans-endothelial activation, but also affects the migration of effector T cells during CRC (Figure 3A) [104]. Moreover, the gene expression profiles in CD4^{+}CD25^{+}Foxp3^{+}CD127^{low} Treg cells was described in patients with CRC. The genetic profiling analysis led to the identification of 61 immune-related genes in Treg cells. Most of these genes were involved in cytokine/chemokine mediators of inflammation, chemokine receptors, lymphocyte activation, and TCR receptor signaling pathways, CCR1, CCR2, IL-10, and SOCS3 [105] being the most relevant. Thus, molecules such as IDO, VEGFR, Runx3, and TGF-β contribute to the promotion of Treg cells during CRC.

6.6. Tumor-Associated Macrophages (TAMs)

TAMs are an important cell component in the microenvironment of various types of solid tumors; in CRC, TAMs are involved in tumor initiation and metastasis [47,48]. Moreover, CD60^{+}INOS^{-} TAMs that infiltrated tumors of CRC patients have been associated with increased CD8^{+}Foxp3^{+} Treg cells in the tumor stroma, being a negative prognostic factor in patients with CRC (Figure 3A) [106]. Moreover, TAMs can recruit CCR6^{+}Treg cells through CCL20 production and promote CRC in an orthotopic mouse model [107]. There are also other cell interactions with Treg cells, for example, Treg cells and mast cells are abundant in both human CRC and murine APC$^{\Delta 468}$ tumors, and the interaction between Treg-mast cells generates a suppressive-inflammatory Treg cell population that produces IL-17, which favors the expansion and degranulation of mast cells [108]. Taken together, these interactions between myeloid cells and Treg cells indicate an unfavorable prognostic.

6.7. Natural Killer (NK) Cells

On the contrary, NK cells have a cytotoxic role during myeloid leukemia, but their role in solid tumors is controversial because NK cells have limited infiltration [109]. It has been shown that CRC patients have an increased percentage of circulating Treg cells and reduced expression of NKp44 and NKp46 on both NK and NKT cells [110]. In chimeric mice lacking T cells and developing spontaneous intestinal tumors, it was demonstrated that the adoptive transfer of Treg cells and the NK cell depletion

6.3. Vascular Endothelial Growth Factor (VEGF)

A molecule involved in Treg cell promotion is VEGF, which is both secreted and abundant in the tumor microenvironment and suppresses anti-tumor immunity. Its receptor, VEGFR2, is expressed selectively in intra-tumor Treg cells with high expression of Foxp3 (Figure 3A) [101], and it is proposed that both VEGFR2 and Foxp3 may be better predictive markers for recurrence and survival in patients with CRC [101].

6.4. Indoleamine 2,3-Dioxygenase (IDO)

In different types of tumors, the IDO enzyme expression has been found to be increased in tumor tissue and in draining lymph nodes, and it is believed that it plays a role in tumor evasion by suppressing the immune response [28]. Ido1$^{-/-}$ mice (Ido1 is a paralog enzyme involved in the degradation of tryptophan) either bred with APC$^{min/+}$ mice or in the CAC model did not lead to significant differences in the size and number of colon tumors. However, Ido1 deficiency altered the immune response in the tumor microenvironment with increased levels of pro-inflammatory cytokines and a reduced number of Treg cells (Figure 3A). Thus, the exclusive elimination of IDO is not sufficient to reduce the progression of colon cancer [102].

6.5. CD39

As previously mentioned, CD39 is an ecto-enzyme that mediates the generation of immunosuppressive adenosine [20,21]. Treg cells from CRC patients express high levels of CD39 [103], which may increase the amount of adenosine available in the tumor microenvironment. Adenosine reduces the capacity of monocytes to activate the endothelium, which indirectly affects T cells' recruitment to reach the site of the tumor. Thus, Treg-derived adenosine acts on monocytes and not only helps reduce trans-endothelial activation, but also affects the migration of effector T cells during CRC (Figure 3A) [104]. Moreover, the gene expression profiles in CD4^{+}CD25^{+}Foxp3^{+}CD127^{low} Treg cells was described in patients with CRC. The genetic profiling analysis led to the identification of 61 immune-related genes in Treg cells. Most of these genes were involved in cytokine/chemokine mediators of inflammation, chemokine receptors, lymphocyte activation, and TCR receptor signaling pathways, CCR1, CCR2, IL-10, and SOCS3 [105] being the most relevant. Thus, molecules such as IDO, VEGFR, Runx3, and TGF-β contribute to the promotion of Treg cells during CRC.

6.6. Tumor-Associated Macrophages (TAMs)

TAMs are an important cell component in the microenvironment of various types of solid tumors; in CRC, TAMs are involved in tumor initiation and metastasis [47,48]. Moreover, CD60^{+}INOS^{-} TAMs that infiltrated tumors of CRC patients have been associated with increased CD8^{+}Foxp3^{+} Treg cells in the tumor stroma, being a negative prognostic factor in patients with CRC (Figure 3A) [106]. Moreover, TAMs can recruit CCR6^{+}Treg cells through CCL20 production and promote CRC in an orthotopic mouse model [107]. There are also other cell interactions with Treg cells, for example, Treg cells and mast cells are abundant in both human CRC and murine APC$^{\Delta 468}$ tumors, and the interaction between Treg-mast cells generates a suppressive-inflammatory Treg cell population that produces IL-17, which favors the expansion and degranulation of mast cells [108]. Taken together, these interactions between myeloid cells and Treg cells indicate an unfavorable prognostic.

6.7. Natural Killer (NK) Cells

On the contrary, NK cells have a cytotoxic role during myeloid leukemia, but their role in solid tumors is controversial because NK cells have limited infiltration [109]. It has been shown that CRC patients have an increased percentage of circulating Treg cells and reduced expression of NKp44 and NKp46 on both NK and NKT cells [110]. In chimeric mice lacking T cells and developing spontaneous intestinal tumors, it was demonstrated that the adoptive transfer of Treg cells and the NK cell depletion

increased dramatically both the number and size of tumors with a decreased survival rate; this correlates with an impaired systemic production of IFN-γ (Figure 3B) [111]. Thus, there probably exists an interference of Treg cells over NK cells during CRC. This was demonstrated recently in a phase-1 clinical trial of adoptive transfer of expanded NK cells in combination with IgG1 antibody in patients with CRC and gastric cancer, in which this combinatory immunotherapy enhanced IFN-γ production and reduced peripheral Treg cells, and some patients showed an overall decrease in tumor size [112].

6.8. Invariant NK T Cells (iNKT Cells)

iNKT cells have an anti-tumor function and participate in the control of tumor metastasis through the secretion of IFN-γ [113]. Controversially, the absence of iNKT cells decreased the number of intestinal polyps in $APC^{min/+}$ mice, correlating with a reduced frequency of Treg cells. Additionally, Ifn-γ and Nos2 genes were increased in polyps with increased frequencies of $CD4^+$ and $CD8^+$ T cells, suggesting that iNKT cells promote polyp formation in the intestine [114]. Different roles between either NK or iNKT cells have been demonstrated during CRC, and both impacted Treg cell activities.

7. Are Treg Cells a Good Target for Immunotherapy during CRC?

Depletion of Treg cells has been a target in clinical trials for some types of cancer with contrasting results. In melanoma, the administration of an IL-2/diphtheria toxin fusion protein to eliminate Treg cells apparently did not eliminate these cells or cause regression of metastatic melanoma [115]. However, the same protein fusion IL-2/diphtheria toxin significantly reduced the number of Treg cells in peripheral blood of patients with metastatic renal carcinoma and abrogated Treg-mediated immunosuppressive activity in vivo [116]. Some basic and clinical trials render strong evidence to show that Treg cells are therapeutic targets during CRC development. For example, low doses of an adenovirus expressing the IL-12 gene mediated a potent anti-tumor effect against subcutaneous colorectal carcinomas in mice in an immunosuppressive environment; this is caused by a direct effect on Treg cells, inhibiting in vitro secretion of IL-10 and TGF-β. Moreover, the treatment with the adenovirus-expressing IL-12 gene decreased the number of myeloid-derived suppressor cells (MDSC) and generated specific $CD4^+IFN\text{-}\gamma^+$ cells that were involved in the eradication of tumors (Figure 3B) [117]. Our own research suggests that Tim-3 is over-expressed in Treg cells in the CAC model [63]. It has also been suggested that $Tim3^+Foxp3^+$ Treg cells represent specialized tumor resident $Foxp3^+$ cells that probably have a role in T cell dysfunction [35,118,119]. Supporting this last idea, it was demonstrated that Tim-3 is expressed on $CD8^+$ tumor-infiltrating lymphocytes isolated from patients with CRC; these $CD8^+$ T cells also co-expressed PD-1 and exhibited an exhausted phenotype because they did not secrete cytokines. Combined targeting of both Tim-3 and PD-1 with monoclonal antibodies increased the frequencies of IFN-γ and TNF-α and the proliferation of antigen-specific $CD8^+$ T cells (Figure 3A). Additionally, with the use of these monoclonal antibodies, a decrease in Treg cells was observed [120]; thus, using either monoclonal antibodies or inhibitor molecules against Tim-3 could induce a protective response against CRC. The LAG-3 receptor is overexpressed in Treg cells from patients with CRC and liver metastasis, and an antibody blockade of LAG-3 increased the proliferation and effector cytokine production of intratumor T cells [121]. Another immunologic target is the CTLA-4, a molecule involved in the negative regulation of activated T cells; in Treg cells, its loss or inhibition results in reduced Treg cell function [122]. In a murine model, the use of anti-CTLA-4 antibody with IgG2a isotype exhibits enhanced antitumor activity in the colon adenocarcinoma tumor model; this effect was associated with a significant reduction in Treg cells at the tumor site, and, consequently, an expansion of activated $CD8^+$ T cells was observed [123]. It has been demonstrated that low doses of cyclophosphamide target Treg cells in humans and animal models. In a clinical trial, 55 patients with metastatic CRC received 2 week-long courses of low-dose cyclophosphamide. An increased number of absolute T-cell numbers was found with a reduction in the percentage and absolute number of Treg, B, and NK cells. In addition, an increased amount of $IFN\text{-}\gamma^+$ tumor-specific T cells and granzyme B were displayed, which was associated with a significant delay in tumor progression (Figure 3B) [124].

Together, these data strongly suggest that Treg cells are an immunotherapeutic target for CRC. We must also consider that immune-targeting Treg cells during CRC may affect some important oncogenes influencing the microenvironment of colorectal tissue such as p53, APC, or Kirsten Rat Sarcoma Viral (KRAS). Some recent studies have shown, for example, that patients with CRC microsatellite stable (MSS) disease do not respond effectively to PD-1 Immune Checkpoint Blockage (ICB), and this is caused by mutations on the oncogene KRAS, which induces the recruitment of MDSC and Treg cells [125]. Tumor cells carrying mutations in KRAS induce highly suppressive Treg cells and over-expression of $KRAS^{G12V}$ gene-induced Treg cells [126]. APC deficiency in the mouse model reduces the presence of Nuclear Factor of Activated cells (NFAT) specifically in Treg cells, reducing differentiation and suppressive capacity [127]. Furthermore, it was suggested that p53 mutations in patients with CRC tumor recurrence is caused by a correlation between p53 and IL-10 from Treg cells [128]. All these observations make it difficult to believe that Treg cells by themselves are a unique suitable therapeutic target. Some studies in murine models and patients attempted to directly or indirectly narrow Treg cells to try to reduce the impact of tumorigenesis in either CRC or gastric cancer development, but these studies have failed because they suggested that CRC tumors are immuno-silent and hypo-responsive to ICB treatment [129–131]. For example, it was demonstrated that during gastric cancer, PD-1 blockade promoted an increased proliferation and suppressive activity of Treg cells [130]. To reduce this negative impact and to improve the efficacy of treatments in solid tumors, we and others have proposed the use of combined immunotherapy. For example, the use of Oxaliplatin (Folfox) in combination with anti-PD-1 antibodies in a murine model of CRC and in samples of patients reduced the number of tumors, which does not happen if these compounds are used individually [132]. The reduction in the number of tumors is caused by $PD\text{-}1^{+}CD8^{+}T\text{-}bet^{+}$ cells infiltrating the tumor. Likewise, the protective immune response in the combined therapy of ICBs with Folfox was confirmed in another murine model of CRC, where the protection was $CD8^{+}$ T-cell-dependent [133]. In the mouse CAC model, it was recently shown that the lack of STAT-6 transcription factor reduced the inflammation and generated protection; however, when STAT-6 activity was inhibited with a chemical drug (AS1517499), a partial, but significant, reduction in tumor development was observed [134]. Consequently, it is clear that a combined therapy using more than one target probably offers improved protection against the tumor generation during CRC, or at least has an increased likelihood of stopping CRC development. In fact, in a recent work targeting STAT-6 together with the inflammatory response and at the same time receiving the classical treatment of the drug 5-Fluorouracil, the authors were able to reverse previously established colon tumors using the CAC model [135]. These findings support the idea that targeting multiple molecules may be the best way to defeat this type of cancer.

8. Conclusions

There is strong evidence supporting the fact that the immune response plays a major role in CRC development, and some immunological interacting mechanisms create a complex network that leads to the development of polyps, adenomas, and tumors. Some years ago, we learnt lessons about complex immunological networks triggered during infectious diseases. We also learnt how to manipulate the host immune system for a better response during vaccine treatment. Now, we are applying these lessons to the development of cancer and, surprisingly, the facts that were described as having a role in the correct adaptation of parasites to the host are very similar to the causes of the development of tumors that lead to cancer. During CRC, tumors promote immune cell populations that favor immunosuppression; thus, tumors can be established without selective pressure. It is necessary to learn how we can manipulate this immunosuppressive microenvironment to choose the correct and timely action to modulate the immune response, which could probably (and hopefully) eradicate the tumor. We also need to learn how the reversion of immunosuppressive microenvironments during cancer would not cause autoimmunity or dangerous inflammatory responses. Here, the participation of Tregs appears to be crucial, but it is still controversial, given that removing Tregs may likely produce one of two disparate results: on the one hand, it could improve the anti-tumor immune response and

accelerate tumor rejection; on the other hand, it could promote tumor establishment by releasing a long-lasting inflammatory response. The series of contrasting reports regarding Tregs in colon cancer described here tell us that we do not have enough information to determine which molecules associated with Tregs should be removed during CRC development, and whether they should be removed at the beginning or at advanced stages of CRC. Perhaps a combination of therapies that focus on different targets in either the same or another subpopulation of immune cells could be a first step to begin to win the battle against CRC.

Author Contributions: J.E.O. collected the data, J.E.O., I.M.-A. and T.R. developed the images, J.E.O., M.R.-S. and L.I.T. wrote the first draft with contributions from I.M.-A. and T.R. All authors reviewed and worked in the final version. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by CONACYT grant number A1-S-37879 and DGAPA-PAPIIT-UNAM grant number IA209720. The APC was funded by CONACYT A1-S-37879.

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

References

1. Wang, M.; Yin, B.; Wang, H.Y.; Wang, R.F. Current advances in T-cell-based cancer immunotherapy. *Immunotherapy* **2014**, *6*, 1265–1278. [CrossRef] [PubMed]
2. Yang, Y. Cancer immunotherapy: Harnessing the immune system to battle cancer. *J. Clin. Investig.* **2015**, *125*, 3335–3337. [CrossRef] [PubMed]
3. Rosenberg, S.A.; Yannelli, J.R.; Yang, J.C.; Topalian, S.L.; Schwartzentruber, D.J.; Weber, J.S.; Parkinson, D.R.; Seipp, C.A.; Einhorn, J.H.; White, D.E. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst.* **1994**, *86*, 1159–1166. [CrossRef] [PubMed]
4. Niccolai, E.; Taddei, A.; Prisco, D.; Amedei, A. Gastric cancer and the epoch of immunotherapy approaches. *World J. Gastroenterol.* **2015**, *21*, 5778–5793. [CrossRef]
5. Berrien-Elliott, M.M.; Romee, R.; Fehniger, T.A. Improving natural killer cell cancer immunotherapy. *Curr. Opin. Organ Transplant.* **2015**, *20*, 671–680. [CrossRef]
6. Anagnostou, V.K.; Brahmer, J.R. Cancer immunotherapy: A future paradigm shift in the treatment of non-small cell lung cancer. *Clin. Cancer Res.* **2015**, *21*, 976–984. [CrossRef]
7. Xiang, B.; Snook, A.E.; Magee, M.S.; Waldman, S.A. Colorectal cancer immunotherapy. *Discov. Med.* **2013**, *15*, 301–308.
8. Vignali, D.A.; Collison, L.W.; Workman, C.J. How regulatory T cells work. *Nat. Rev. Immunol.* **2008**, *8*, 523–532. [CrossRef]
9. Sakaguchi, S.; Sakaguchi, N.; Asano, M.; Itoh, M.; Toda, M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **1995**, *155*, 1151–1164.
10. Ashwell, J.D.; Robb, R.J.; Malek, T.R. Proliferation of T lymphocytes in response to interleukin 2 varies with their state of activation. *J. Immunol.* **1986**, *137*, 2572–2578.
11. Lenardo, M.J. Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature* **1991**, *353*, 858–861. [CrossRef] [PubMed]
12. Fontenot, J.D.; Gavin, M.A.; Rudensky, A.Y. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat. Immunol.* **2003**, *4*, 330–336. [CrossRef] [PubMed]
13. Hori, S.; Nomura, T.; Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **2003**, *299*, 1057–1061. [CrossRef] [PubMed]
14. Josefowicz, S.Z.; Lu, L.F.; Rudensky, A.Y. Regulatory T cells: Mechanisms of differentiation and function. *Annu. Rev. Immunol.* **2012**, *30*, 531–564. [CrossRef]
15. Gambineri, E.; Torgerson, T.R.; Ochs, H.D. Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Curr. Opin. Rheumatol.* **2003**, *15*, 430–435. [CrossRef]
16. Fontenot, J.D.; Rasmussen, J.P.; Gavin, M.A.; Rudensky, A.Y. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat. Immunol.* **2005**, *6*, 1142–1151. [CrossRef]

17. Pandiyan, P.; Zheng, L.; Ishihara, S.; Reed, J.; Lenardo, M.J. $CD4^+CD25^+Foxp^{3+}$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^+$ T cells. *Nat. Immunol.* **2007**, *8*, 1353–1362. [CrossRef]
18. Salinas, N.; Olguin, J.E.; Castellanos, C.; Saavedra, R. T cell suppression in vitro during Toxoplasma gondii infection is the result of IL-2 competition between Tregs and T cells leading to death of proliferating T cells. *Scand. J. Immunol.* **2014**, *79*, 1–11. [CrossRef]
19. Setoguchi, R.; Hori, S.; Takahashi, T.; Sakaguchi, S. Homeostatic maintenance of natural $Foxp3^+$ $CD25^+$ $CD4^+$ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J. Exp. Med.* **2005**, *201*, 723–735. [CrossRef]
20. Borsellino, G.; Kleinewietfeld, M.; Di Mitri, D.; Sternjak, A.; Diamantini, A.; Giometto, R.; Hopner, S.; Centonze, D.; Bernardi, G.; Dell'Acqua, M.L.; et al. Expression of ectonucleotidase CD39 by $Foxp3^+$ Treg cells: Hydrolysis of extracellular ATP and immune suppression. *Blood* **2007**, *110*, 1225–1232. [CrossRef] [PubMed]
21. Deaglio, S.; Dwyer, K.M.; Gao, W.; Friedman, D.; Usheva, A.; Erat, A.; Chen, J.F.; Enjyoji, K.; Linden, J.; Oukka, M.; et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J. Exp. Med.* **2007**, *204*, 1257–1265. [CrossRef] [PubMed]
22. Kobie, J.J.; Shah, P.R.; Yang, L.; Rebhahn, J.A.; Fowell, D.J.; Mosmann, T.R. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5′-adenosine monophosphate to adenosine. *J. Immunol.* **2006**, *177*, 6780–6786. [CrossRef] [PubMed]
23. Saraiva, M.; O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **2010**, *10*, 170–181. [CrossRef] [PubMed]
24. Huber, S.; Schramm, C.; Lehr, H.A.; Mann, A.; Schmitt, S.; Becker, C.; Protschka, M.; Galle, P.R.; Neurath, M.F.; Blessing, M. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory $CD4^+CD25^+$ T cells. *J. Immunol.* **2004**, *173*, 6526–6531. [CrossRef] [PubMed]
25. Nakamura, K.; Kitani, A.; Fuss, I.; Pedersen, A.; Harada, N.; Nawata, H.; Strober, W. TGF-beta 1 plays an important role in the mechanism of $CD4^+CD25^+$ regulatory T cell activity in both humans and mice. *J. Immunol.* **2004**, *172*, 834–842. [CrossRef]
26. Collison, L.W.; Pillai, M.R.; Chaturvedi, V.; Vignali, D.A. Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J. Immunol.* **2009**, *182*, 6121–6128. [CrossRef] [PubMed]
27. Collison, L.W.; Workman, C.J.; Kuo, T.T.; Boyd, K.; Wang, Y.; Vignali, K.M.; Cross, R.; Sehy, D.; Blumberg, R.S.; Vignali, D.A. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* **2007**, *450*, 566–569. [CrossRef]
28. Mellor, A.L.; Munn, D.H. IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* **2004**, *4*, 762–774. [CrossRef]
29. Schneider, H.; Valk, E.; da Rocha Dias, S.; Wei, B.; Rudd, C.E. CTLA-4 up-regulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 12861–12866. [CrossRef]
30. Tang, Q.; Boden, E.K.; Henriksen, K.J.; Bour-Jordan, H.; Bi, M.; Bluestone, J.A. Distinct roles of CTLA-4 and TGF-beta in $CD4^+CD25^+$ regulatory T cell function. *Eur. J. Immunol.* **2004**, *34*, 2996–3005. [CrossRef]
31. Cao, X.; Cai, S.F.; Fehniger, T.A.; Song, J.; Collins, L.I.; Piwnica-Worms, D.R.; Ley, T.J. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* **2007**, *27*, 635–646. [CrossRef]
32. Grossman, W.J.; Verbsky, J.W.; Barchet, W.; Colonna, M.; Atkinson, J.P.; Ley, T.J. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **2004**, *21*, 589–601. [CrossRef]
33. Miyara, M.; Sakaguchi, S. Natural regulatory T cells: Mechanisms of suppression. *Trends Mol. Med.* **2007**, *13*, 108–116. [CrossRef]
34. Bardhan, K.; Anagnostou, T.; Boussiotis, V.A. The PD1:PD-L1/2 Pathway from Discovery to Clinical Implementation. *Front. Immunol.* **2016**, *7*, 550. [CrossRef]
35. Sakuishi, K.; Ngiow, S.F.; Sullivan, J.M.; Teng, M.W.; Kuchroo, V.K.; Smyth, M.J.; Anderson, A.C. $TIM3^+FOXP3^+$ regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. *Oncoimmunology* **2013**, *2*, e23849. [CrossRef]

36. Tariq, K.; Ghias, K. Colorectal cancer carcinogenesis: A review of mechanisms. *Cancer Biol. Med.* **2016**, *13*, 120–135. [CrossRef]
37. Sharma, R. An examination of colorectal cancer burden by socioeconomic status: Evidence from GLOBOCAN 2018. *EPMA J.* **2020**, *11*, 95–117. [CrossRef]
38. Jemal, A.; Center, M.M.; DeSantis, C.; Ward, E.M. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 1893–1907. [CrossRef]
39. Jemal, A.; Siegel, R.; Xu, J.; Ward, E. Cancer statistics, 2010. *CA Cancer J. Clin.* **2010**, *60*, 277–300. [CrossRef]
40. Siegel, R.; Desantis, C.; Jemal, A. Colorectal cancer statistics, 2014. *CA Cancer J. Clin.* **2014**, *64*, 104–117. [CrossRef]
41. Jess, T.; Frisch, M.; Simonsen, J. Trends in overall and cause-specific mortality among patients with inflammatory bowel disease from 1982 to 2010. *Clin. Gastroenterol. Hepatol.* **2013**, *11*, 43–48. [CrossRef]
42. Terzic, J.; Grivennikov, S.; Karin, E.; Karin, M. Inflammation and colon cancer. *Gastroenterology* **2010**, *138*, 2101–2114.e5. [CrossRef]
43. Resch, A.; Langner, C. Risk assessment in early colorectal cancer: Histological and molecular markers. *Dig. Dis.* **2015**, *33*, 77–85. [CrossRef]
44. Rooney, M.S.; Shukla, S.A.; Wu, C.J.; Getz, G.; Hacohen, N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* **2015**, *160*, 48–61. [CrossRef]
45. de Vries, N.L.; Swets, M.; Vahrmeijer, A.L.; Hokland, M.; Kuppen, P.J. The Immunogenicity of Colorectal Cancer in Relation to Tumor Development and Treatment. *Int. J. Mol. Sci.* **2016**, *17*, 1030. [CrossRef]
46. Schreiber, R.D.; Old, L.J.; Smyth, M.J. Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion. *Science* **2011**, *331*, 1565–1570. [CrossRef]
47. Biswas, S.K.; Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat. Immunol.* **2010**, *11*, 889–896. [CrossRef]
48. Galdiero, M.R.; Bonavita, E.; Barajon, I.; Garlanda, C.; Mantovani, A.; Jaillon, S. Tumor associated macrophages and neutrophils in cancer. *Immunobiology* **2013**, *218*, 1402–1410. [CrossRef]
49. Ling, Z.A.; Zhang, L.J.; Ye, Z.H.; Dang, Y.W.; Chen, G.; Li, R.L.; Zeng, J.J. Immunohistochemical distribution of FOXP3$^+$ regulatory T cells in colorectal cancer patients. *Int. J. Clin. Exp. Pathol.* **2018**, *11*, 1841–1854.
50. Ma, Q.; Liu, J.; Wu, G.; Teng, M.; Wang, S.; Cui, M.; Li, Y. Co-expression of LAG3 and TIM3 identifies a potent Treg population that suppresses macrophage functions in colorectal cancer patients. *Clin. Exp. Pharmacol. Physiol.* **2018**, *45*, 1002–1009. [CrossRef]
51. Norton, S.E.; Ward-Hartstonge, K.A.; McCall, J.L.; Leman, J.K.H.; Taylor, E.S.; Munro, F.; Black, M.A.; Fazekas de St Groth, B.; McGuire, H.M.; Kemp, R.A. High-Dimensional Mass Cytometric Analysis Reveals an Increase in Effector Regulatory T Cells as a Distinguishing Feature of Colorectal Tumors. *J. Immunol.* **2019**, *202*, 1871–1884. [CrossRef]
52. Loddenkemper, C.; Schernus, M.; Noutsias, M.; Stein, H.; Thiel, E.; Nagorsen, D. In situ analysis of FOXP3$^+$ regulatory T cells in human colorectal cancer. *J. Transl. Med.* **2006**, *4*, 52. [CrossRef]
53. Xu, W.; Liu, H.; Song, J.; Fu, H.X.; Qiu, L.; Zhang, B.F.; Li, H.Z.; Bai, J.; Zheng, J.N. The appearance of Tregs in cancer nest is a promising independent risk factor in colon cancer. *J. Cancer Res. Clin. Oncol.* **2013**, *139*, 1845–1852. [CrossRef]
54. Liu, Z.; Huang, Q.; Liu, G.; Dang, L.; Chu, D.; Tao, K.; Wang, W. Presence of FOXP3$^+$Treg cells is correlated with colorectal cancer progression. *Int. J. Clin. Exp. Med.* **2014**, *7*, 1781–1785.
55. Clarke, S.L.; Betts, G.J.; Plant, A.; Wright, K.L.; El-Shanawany, T.M.; Harrop, R.; Torkington, J.; Rees, B.I.; Williams, G.T.; Gallimore, A.M.; et al. CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. *PLoS ONE* **2006**, *1*, e129. [CrossRef]
56. Deng, L.; Zhang, H.; Luan, Y.; Zhang, J.; Xing, Q.; Dong, S.; Wu, X.; Liu, M.; Wang, S. Accumulation of foxp3$^+$ T regulatory cells in draining lymph nodes correlates with disease progression and immune suppression in colorectal cancer patients. *Clin. Cancer Res.* **2010**, *16*, 4105–4112. [CrossRef]
57. Fujimoto, H.; Saito, Y.; Ohuchida, K.; Kawakami, E.; Fujiki, S.; Watanabe, T.; Ono, R.; Kaneko, A.; Takagi, S.; Najima, Y.; et al. Deregulated Mucosal Immune Surveillance through Gut-Associated Regulatory T Cells and PD-1$^+$ T Cells in Human Colorectal Cancer. *J. Immunol.* **2018**, *200*, 3291–3303. [CrossRef]
58. Bonertz, A.; Weitz, J.; Pietsch, D.H.; Rahbari, N.N.; Schlude, C.; Ge, Y.; Juenger, S.; Vlodavsky, I.; Khazaie, K.; Jaeger, D.; et al. Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. *J. Clin. Investig.* **2009**, *119*, 3311–3321. [CrossRef] [PubMed]

59. Tanaka, T.; Kohno, H.; Suzuki, R.; Yamada, Y.; Sugie, S.; Mori, H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.* **2003**, *94*, 965–973. [CrossRef]
60. Pastille, E.; Bardini, K.; Fleissner, D.; Adamczyk, A.; Frede, A.; Wadwa, M.; von Smolinski, D.; Kasper, S.; Sparwasser, T.; Gruber, A.D.; et al. Transient ablation of regulatory T cells improves antitumor immunity in colitis-associated colon cancer. *Cancer Res.* **2014**, *74*, 4258–4269. [CrossRef]
61. Grimmig, T.; Kim, M.; Germer, C.T.; Gasser, M.; Waaga-Gasser, A.M. The role of FOXP3 in disease progression in colorectal cancer patients. *Oncoimmunology* **2013**, *2*, e24521. [CrossRef] [PubMed]
62. Lahl, K.; Loddenkemper, C.; Drouin, C.; Freyer, J.; Arnason, J.; Eberl, G.; Hamann, A.; Wagner, H.; Huehn, J.; Sparwasser, T. Selective depletion of Foxp3^{+} regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* **2007**, *204*, 57–63. [CrossRef] [PubMed]
63. Olguin, J.E.; Medina-Andrade, I.; Molina, E.; Vazquez, A.; Pacheco-Fernandez, T.; Saavedra, R.; Perez-Plasencia, C.; Chirino, Y.I.; Vaca-Paniagua, F.; Arias-Romero, L.E.; et al. Early and Partial Reduction in CD4^{+}Foxp3^{+} Regulatory T Cells during Colitis-Associated Colon Cancer Induces CD4^{+} and CD8^{+} T Cell Activation Inhibiting Tumorigenesis. *J. Cancer* **2018**, *9*, 239–249. [CrossRef]
64. Kazama, K.; Otake, J.; Satoyoshi, T.; Shiozawa, M.; Sugano, N.; Sato, S.; Atsumi, Y.; Kano, K.; Murakawa, M.; Maezawa, Y.; et al. Distribution of Regulatory T-Cells and Other Phenotypes of T-Cells in Tumors and Regional Lymph Nodes of Colorectal Cancer Patients. *In Vivo* **2020**, *34*, 849–856. [CrossRef]
65. James, E.; Yeh, A.; King, C.; Korangy, F.; Bailey, I.; Boulanger, D.S.; Van den Eynde, B.J.; Murray, N.; Elliott, T.J. Differential suppression of tumor-specific CD8^{+} T cells by regulatory T cells. *J. Immunol.* **2010**, *185*, 5048–5055. [CrossRef]
66. Stagg, J.; Divisekera, U.; Duret, H.; Sparwasser, T.; Teng, M.W.; Darcy, P.K.; Smyth, M.J. CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res.* **2011**, *71*, 2892–2900. [CrossRef] [PubMed]
67. Moser, A.R.; Pitot, H.C.; Dove, W.F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* **1990**, *247*, 322–324. [CrossRef]
68. Niho, N.; Takahashi, M.; Kitamura, T.; Shoji, Y.; Itoh, M.; Noda, T.; Sugimura, T.; Wakabayashi, K. Concomitant suppression of hyperlipidemia and intestinal polyp formation in Apc-deficient mice by peroxisome proliferator-activated receptor ligands. *Cancer Res.* **2003**, *63*, 6090–6095.
69. Akeus, P.; Langenes, V.; von Mentzer, A.; Yrlid, U.; Sjoling, A.; Saksena, P.; Raghavan, S.; Quiding-Jarbrink, M. Altered chemokine production and accumulation of regulatory T cells in intestinal adenomas of APC(Min/+) mice. *Cancer Immunol. Immunother. CII* **2014**, *63*, 807–819. [CrossRef]
70. Akeus, P.; Langenes, V.; Kristensen, J.; von Mentzer, A.; Sparwasser, T.; Raghavan, S.; Quiding-Jarbrink, M. Treg-cell depletion promotes chemokine production and accumulation of CXCR3^{+} conventional T cells in intestinal tumors. *Eur. J. Immunol.* **2015**, *45*, 1654–1666. [CrossRef]
71. Akeus, P.; Szeponik, L.; Ahlmanner, F.; Sundstrom, P.; Alsen, S.; Gustavsson, B.; Sparwasser, T.; Raghavan, S.; Quiding-Jarbrink, M. Regulatory T cells control endothelial chemokine production and migration of T cells into intestinal tumors of APC(min/+) mice. *Cancer Immunol. Immunother. CII* **2018**, *67*, 1067–1077. [CrossRef] [PubMed]
72. Abron, J.D.; Singh, N.P.; Murphy, A.E.; Mishra, M.K.; Price, R.L.; Nagarkatti, M.; Nagarkatti, P.S.; Singh, U.P. Differential role of CXCR3 in inflammation and colorectal cancer. *Oncotarget* **2018**, *9*, 17928–17936. [CrossRef] [PubMed]
73. Chung, A.Y.; Li, Q.; Blair, S.J.; De Jesus, M.; Dennis, K.L.; LeVea, C.; Yao, J.; Sun, Y.; Conway, T.F.; Virtuoso, L.P.; et al. Oral interleukin-10 alleviates polyposis via neutralization of pathogenic T-regulatory cells. *Cancer Res.* **2014**, *74*, 5377–5385. [CrossRef]
74. Szeponik, L.; Akeus, P.; Rodin, W.; Raghavan, S.; Quiding-Jarbrink, M. Regulatory T cells specifically suppress conventional CD8alphabeta T cells in intestinal tumors of APC(Min/+) mice. *Cancer Immunol. Immunother. CII* **2020**. [CrossRef] [PubMed]
75. Dennis, K.L.; Wang, Y.; Blatner, N.R.; Wang, S.; Saadalla, A.; Trudeau, E.; Roers, A.; Weaver, C.T.; Lee, J.J.; Gilbert, J.A.; et al. Adenomatous polyps are driven by microbe-instigated focal inflammation and are controlled by IL-10-producing T cells. *Cancer Res.* **2013**, *73*, 5905–5913. [CrossRef] [PubMed]
76. Romagnani, S. The Th1/Th2 paradigm. *Immunol. Today* **1997**, *18*, 263–266. [CrossRef]

77. Lee, Y.K.; Mukasa, R.; Hatton, R.D.; Weaver, C.T. Developmental plasticity of Th17 and Treg cells. *Curr. Opin. Immunol.* **2009**, *21*, 274–280. [CrossRef]
78. O'Shea, J.J.; Paul, W.E. Mechanisms underlying lineage commitment and plasticity of helper CD4$^+$ T cells. *Science* **2010**, *327*, 1098–1102. [CrossRef] [PubMed]
79. Galli, S.J.; Borregaard, N.; Wynn, T.A. Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nat. Immunol.* **2011**, *12*, 1035–1044. [CrossRef]
80. Ma, C.; Dong, X. Colorectal cancer-derived Foxp3$^+$ IL-17$^+$ T cells suppress tumour-specific CD8$^+$ T cells. *Scand. J. Immunol.* **2011**, *74*, 47–51. [CrossRef]
81. Kryczek, I.; Wu, K.; Zhao, E.; Wei, S.; Vatan, L.; Szeliga, W.; Huang, E.; Greenson, J.; Chang, A.; Rolinski, J.; et al. IL-17$^+$ regulatory T cells in the microenvironments of chronic inflammation and cancer. *J. Immunol.* **2011**, *186*, 4388–4395. [CrossRef] [PubMed]
82. Li, L.; Boussiotis, V.A. The role of IL-17-producing Foxp3$^+$ CD4$^+$ T cells in inflammatory bowel disease and colon cancer. *Clin. Immunol.* **2013**, *148*, 246–253. [CrossRef] [PubMed]
83. Rizzo, A.; Di Giovangiulio, M.; Stolfi, C.; Franze, E.; Fehling, H.J.; Carsetti, R.; Giorda, E.; Colantoni, A.; Ortenzi, A.; Rugge, M.; et al. RORgammat-Expressing Tregs Drive the Growth of Colitis-Associated Colorectal Cancer by Controlling IL6 in Dendritic Cells. *Cancer Immunol. Res.* **2018**, *6*, 1082–1092. [CrossRef] [PubMed]
84. Blatner, N.R.; Mulcahy, M.F.; Dennis, K.L.; Scholtens, D.; Bentrem, D.J.; Phillips, J.D.; Ham, S.; Sandall, B.P.; Khan, M.W.; Mahvi, D.M.; et al. Expression of RORgammat marks a pathogenic regulatory T cell subset in human colon cancer. *Sci. Transl. Med.* **2012**, *4*, 164ra159. [CrossRef] [PubMed]
85. Keerthivasan, S.; Aghajani, K.; Dose, M.; Molinero, L.; Khan, M.W.; Venkateswaran, V.; Weber, C.; Emmanuel, A.O.; Sun, T.; Bentrem, D.J.; et al. beta-Catenin promotes colitis and colon cancer through imprinting of proinflammatory properties in T cells. *Sci. Transl. Med.* **2014**, *6*, 225ra228. [CrossRef] [PubMed]
86. Ward-Hartstonge, K.A.; McCall, J.L.; McCulloch, T.R.; Kamps, A.K.; Girardin, A.; Cretney, E.; Munro, F.M.; Kemp, R.A. Inclusion of BLIMP-1$^+$ effector regulatory T cells improves the Immunoscore in a cohort of New Zealand colorectal cancer patients: A pilot study. *Cancer Immunol. Immunother. CII* **2017**, *66*, 515–522. [CrossRef] [PubMed]
87. Saito, T.; Nishikawa, H.; Wada, H.; Nagano, Y.; Sugiyama, D.; Atarashi, K.; Maeda, Y.; Hamaguchi, M.; Ohkura, N.; Sato, E.; et al. Two FOXP3$^+$CD4$^+$ T cell subpopulations distinctly control the prognosis of colorectal cancers. *Nat. Med.* **2016**, *22*, 679–684. [CrossRef]
88. Scurr, M.; Ladell, K.; Besneux, M.; Christian, A.; Hockey, T.; Smart, K.; Bridgeman, H.; Hargest, R.; Phillips, S.; Davies, M.; et al. Highly prevalent colorectal cancer-infiltrating LAP$^+$ Foxp3$^-$ T cells exhibit more potent immunosuppressive activity than Foxp3$^+$ regulatory T cells. *Mucosal Immunol.* **2014**, *7*, 428–439. [CrossRef]
89. Shevach, E.M.; Thornton, A.M. tTregs, pTregs, and iTregs: Similarities and differences. *Immunol. Rev.* **2014**, *259*, 88–102. [CrossRef]
90. Syed Khaja, A.S.; Toor, S.M.; El Salhat, H.; Ali, B.R.; Elkord, E. Intratumoral FoxP3$^+$Helios$^+$ Regulatory T Cells Upregulating Immunosuppressive Molecules Are Expanded in Human Colorectal Cancer. *Front. Immunol.* **2017**, *8*, 619. [CrossRef]
91. Ma, Y.; Chen, L.; Xie, G.; Zhou, Y.; Yue, C.; Yuan, X.; Zheng, Y.; Wang, W.; Deng, L.; Shen, L. Elevated level of interleukin-35 in colorectal cancer induces conversion of T cells into iTr35 by activating STAT1/STAT3. *Oncotarget* **2016**, *7*, 73003–73015. [CrossRef] [PubMed]
92. Chang, L.Y.; Lin, Y.C.; Mahalingam, J.; Huang, C.T.; Chen, T.W.; Kang, C.W.; Peng, H.M.; Chu, Y.Y.; Chiang, J.M.; Dutta, A.; et al. Tumor-derived chemokine CCL5 enhances TGF-beta-mediated killing of CD8$^+$ T cells in colon cancer by T-regulatory cells. *Cancer Res.* **2012**, *72*, 1092–1102. [CrossRef] [PubMed]
93. Ward, S.T.; Li, K.K.; Hepburn, E.; Weston, C.J.; Curbishley, S.M.; Reynolds, G.M.; Hejmadi, R.K.; Bicknell, R.; Eksteen, B.; Ismail, T.; et al. The effects of CCR5 inhibition on regulatory T-cell recruitment to colorectal cancer. *Br. J. Cancer* **2015**, *112*, 319–328. [CrossRef] [PubMed]
94. Markowitz, S.; Wang, J.; Myeroff, L.; Parsons, R.; Sun, L.; Lutterbaugh, J.; Fan, R.S.; Zborowska, E.; Kinzler, K.W.; Vogelstein, B.; et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science* **1995**, *268*, 1336–1338. [CrossRef]
95. Taniuchi, I.; Osato, M.; Egawa, T.; Sunshine, M.J.; Bae, S.C.; Komori, T.; Ito, Y.; Littman, D.R. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* **2002**, *111*, 621–633. [CrossRef]

96. Sato, T.; Ohno, S.; Hayashi, T.; Sato, C.; Kohu, K.; Satake, M.; Habu, S. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* **2005**, *22*, 317–328. [CrossRef]
97. Sugai, M.; Aoki, K.; Osato, M.; Nambu, Y.; Ito, K.; Taketo, M.M.; Shimizu, A. Runx3 is required for full activation of regulatory T cells to prevent colitis-associated tumor formation. *J. Immunol.* **2011**, *186*, 6515–6520. [CrossRef]
98. Salem, M.; Wallace, C.; Velegraki, M.; Li, A.; Ansa-Addo, E.; Metelli, A.; Kwon, H.; Riesenberg, B.; Wu, B.; Zhang, Y.; et al. GARP Dampens Cancer Immunity by Sustaining Function and Accumulation of Regulatory T Cells in the Colon. *Cancer Res.* **2019**, *79*, 1178–1190. [CrossRef]
99. Hatzioannou, A.; Banos, A.; Sakelaropoulos, T.; Fedonidis, C.; Vidali, M.S.; Kohne, M.; Handler, K.; Boon, L.; Henriques, A.; Koliaraki, V.; et al. An intrinsic role of IL-33 in Treg cell-mediated tumor immunoevasion. *Nat. Immunol.* **2020**, *21*, 75–85. [CrossRef]
100. Cui, G.; Yuan, A.; Li, Z.; Goll, R.; Florholmen, J. ST2 and regulatory T cells in the colorectal adenoma/carcinoma microenvironment: Implications for diseases progression and prognosis. *Sci. Rep.* **2020**, *10*, 5892. [CrossRef]
101. Suzuki, H.; Onishi, H.; Morisaki, T.; Tanaka, M.; Katano, M. Intratumoral FOXP3$^+$VEGFR2$^+$ regulatory T cells are predictive markers for recurrence and survival in patients with colorectal cancer. *Clin. Immunol.* **2013**, *146*, 26–33. [CrossRef] [PubMed]
102. Takamatsu, M.; Hirata, A.; Ohtaki, H.; Hoshi, M.; Ando, T.; Ito, H.; Hatano, Y.; Tomita, H.; Kuno, T.; Saito, K.; et al. Inhibition of indoleamine 2,3-dioxygenase 1 expression alters immune response in colon tumor microenvironment in mice. *Cancer Sci.* **2015**, *106*, 1008–1015. [CrossRef] [PubMed]
103. Strasser, K.; Birnleitner, H.; Beer, A.; Pils, D.; Gerner, M.C.; Schmetterer, K.G.; Bachleitner-Hofmann, T.; Stift, A.; Bergmann, M.; Oehler, R. Immunological differences between colorectal cancer and normal mucosa uncover a prognostically relevant immune cell profile. *Oncoimmunology* **2019**, *8*, e1537693. [CrossRef] [PubMed]
104. Sundstrom, P.; Stenstad, H.; Langenes, V.; Ahlmanner, F.; Theander, L.; Ndah, T.G.; Fredin, K.; Borjesson, L.; Gustavsson, B.; Bastid, J.; et al. Regulatory T Cells from Colon Cancer Patients Inhibit Effector T-cell Migration through an Adenosine-Dependent Mechanism. *Cancer Immunol. Res.* **2016**, *4*, 183–193. [CrossRef] [PubMed]
105. Johdi, N.A.; Ait-Tahar, K.; Sagap, I.; Jamal, R. Molecular Signatures of Human Regulatory T Cells in Colorectal Cancer and Polyps. *Front. Immunol.* **2017**, *8*, 620. [CrossRef]
106. Waniczek, D.; Lorenc, Z.; Snietura, M.; Wesecki, M.; Kopec, A.; Muc-Wierzgon, M. Tumor-Associated Macrophages and Regulatory T Cells Infiltration and the Clinical Outcome in Colorectal Cancer. *Arch. Immunol. Ther. Exp.* **2017**, *65*, 445–454. [CrossRef]
107. Liu, J.; Zhang, N.; Li, Q.; Zhang, W.; Ke, F.; Leng, Q.; Wang, H.; Chen, J.; Wang, H. Tumor-associated macrophages recruit CCR6$^+$ regulatory T cells and promote the development of colorectal cancer via enhancing CCL20 production in mice. *PLoS ONE* **2011**, *6*, e19495. [CrossRef]
108. Blatner, N.R.; Bonertz, A.; Beckhove, P.; Cheon, E.C.; Krantz, S.B.; Strouch, M.; Weitz, J.; Koch, M.; Halverson, A.L.; Bentrem, D.J.; et al. In colorectal cancer mast cells contribute to systemic regulatory T-cell dysfunction. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 6430–6435. [CrossRef]
109. Coppola, A.; Arriga, R.; Lauro, D.; Del Principe, M.I.; Buccisano, F.; Maurillo, L.; Palomba, P.; Venditti, A.; Sconocchia, G. NK Cell Inflammation in the Clinical Outcome of Colorectal Carcinoma. *Front. Med.* **2015**, *2*, 33. [CrossRef]
110. Krijgsman, D.; de Vries, N.L.; Skovbo, A.; Andersen, M.N.; Swets, M.; Bastiaannet, E.; Vahrmeijer, A.L.; van de Velde, C.J.H.; Heemskerk, M.H.M.; Hokland, M.; et al. Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: The peripheral blood immune cell profile. *Cancer Immunol. Immunother. CII* **2019**, *68*, 1011–1024. [CrossRef]
111. Janakiram, N.B.; Mohammed, A.; Bryant, T.; Brewer, M.; Biddick, L.; Lightfoot, S.; Lang, M.L.; Rao, C.V. Adoptive transfer of regulatory T cells promotes intestinal tumorigenesis and is associated with decreased NK cells and IL-22 binding protein. *Mol. Carcinog.* **2015**, *54*, 986–998. [CrossRef]
112. Ishikawa, T.; Okayama, T.; Sakamoto, N.; Ideno, M.; Oka, K.; Enoki, T.; Mineno, J.; Yoshida, N.; Katada, K.; Kamada, K.; et al. Phase I clinical trial of adoptive transfer of expanded natural killer cells in combination with IgG1 antibody in patients with gastric or colorectal cancer. *Int. J. Cancer* **2018**, *142*, 2599–2609. [CrossRef] [PubMed]

113. Bellone, M.; Ceccon, M.; Grioni, M.; Jachetti, E.; Calcinotto, A.; Napolitano, A.; Freschi, M.; Casorati, G.; Dellabona, P. iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells. *PLoS ONE* **2010**, *5*, e8646. [CrossRef] [PubMed]
114. Wang, Y.; Sedimbi, S.; Lofbom, L.; Singh, A.K.; Porcelli, S.A.; Cardell, S.L. Unique invariant natural killer T cells promote intestinal polyps by suppressing TH1 immunity and promoting regulatory T cells. *Mucosal Immunol.* **2018**, *11*, 131–143. [CrossRef] [PubMed]
115. Attia, P.; Maker, A.V.; Haworth, L.R.; Rogers-Freezer, L.; Rosenberg, S.A. Inability of a fusion protein of IL-2 and diphtheria toxin (Denileukin Diftitox, DAB389IL-2, ONTAK) to eliminate regulatory T lymphocytes in patients with melanoma. *J. Immunother.* **2005**, *28*, 582–592. [CrossRef] [PubMed]
116. Dannull, J.; Su, Z.; Rizzieri, D.; Yang, B.K.; Coleman, D.; Yancey, D.; Zhang, A.; Dahm, P.; Chao, N.; Gilboa, E.; et al. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J. Clin. Investig.* **2005**, *115*, 3623–3633. [CrossRef]
117. Malvicini, M.; Ingolotti, M.; Piccioni, F.; Garcia, M.; Bayo, J.; Atorrasagasti, C.; Alaniz, L.; Aquino, J.B.; Espinoza, J.A.; Gidekel, M.; et al. Reversal of gastrointestinal carcinoma-induced immunosuppression and induction of antitumoural immunity by a combination of cyclophosphamide and gene transfer of IL-12. *Mol. Oncol.* **2011**, *5*, 242–255. [CrossRef]
118. Das, M.; Zhu, C.; Kuchroo, V.K. Tim-3 and its role in regulating anti-tumor immunity. *Immunol. Rev.* **2017**, *276*, 97–111. [CrossRef]
119. Sakuishi, K.; Apetoh, L.; Sullivan, J.M.; Blazar, B.R.; Kuchroo, V.K.; Anderson, A.C. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J. Exp. Med.* **2010**, *207*, 2187–2194. [CrossRef]
120. Liu, J.; Zhang, S.; Hu, Y.; Yang, Z.; Li, J.; Liu, X.; Deng, L.; Wang, Y.; Zhang, X.; Jiang, T.; et al. Targeting PD-1 and Tim-3 Pathways to Reverse CD8 T-Cell Exhaustion and Enhance Ex Vivo T-Cell Responses to Autologous Dendritic/Tumor Vaccines. *J. Immunother.* **2016**, *39*, 171–180. [CrossRef]
121. Zhou, G.; Noordam, L.; Sprengers, D.; Doukas, M.; Boor, P.P.C.; van Beek, A.A.; Erkens, R.; Mancham, S.; Grunhagen, D.; Menon, A.G.; et al. Blockade of LAG3 enhances responses of tumor-infiltrating T cells in mismatch repair-proficient liver metastases of colorectal cancer. *Oncoimmunology* **2018**, *7*, e1448332. [CrossRef] [PubMed]
122. Wing, K.; Onishi, Y.; Prieto-Martin, P.; Yamaguchi, T.; Miyara, M.; Fehervari, Z.; Nomura, T.; Sakaguchi, S. CTLA-4 control over Foxp3^{+} regulatory T cell function. *Science* **2008**, *322*, 271–275. [CrossRef]
123. Selby, M.J.; Engelhardt, J.J.; Quigley, M.; Henning, K.A.; Chen, T.; Srinivasan, M.; Korman, A.J. Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells. *Cancer Immunol. Res.* **2013**, *1*, 32–42. [CrossRef]
124. Scurr, M.; Pembroke, T.; Bloom, A.; Roberts, D.; Thomson, A.; Smart, K.; Bridgeman, H.; Adams, R.; Brewster, A.; Jones, R.; et al. Low-Dose Cyclophosphamide Induces Antitumor T-Cell Responses, which Associate with Survival in Metastatic Colorectal Cancer. *Clin. Cancer Res.* **2017**, *23*, 6771–6780. [CrossRef] [PubMed]
125. Hanggi, K.; Ruffell, B. Oncogenic KRAS Drives Immune Suppression in Colorectal Cancer. *Cancer Cell* **2019**, *35*, 535–537. [CrossRef]
126. Zdanov, S.; Mandapathil, M.; Abu Eid, R.; Adamson-Fadeyi, S.; Wilson, W.; Qian, J.; Carnie, A.; Tarasova, N.; Mkrtichyan, M.; Berzofsky, J.A.; et al. Mutant KRAS Conversion of Conventional T Cells into Regulatory T Cells. *Cancer Immunol. Res.* **2016**, *4*, 354–365. [CrossRef] [PubMed]
127. Aguera-Gonzalez, S.; Burton, O.T.; Vazquez-Chavez, E.; Cuche, C.; Herit, F.; Bouchet, J.; Lasserre, R.; Del Rio-Iniguez, I.; Di Bartolo, V.; Alcover, A. Adenomatous Polyposis Coli Defines Treg Differentiation and Anti-inflammatory Function through Microtubule-Mediated NFAT Localization. *Cell Rep.* **2017**, *21*, 181–194. [CrossRef] [PubMed]
128. Bueter, M.; Gasser, M.; Schramm, N.; Lebedeva, T.; Tocco, G.; Gerstlauer, C.; Grimm, M.; Nichiporuk, E.; Thalheimer, A.; Thiede, A.; et al. T-cell response to p53 tumor-associated antigen in patients with colorectal carcinoma. *Int. J. Oncol.* **2006**, *28*, 431–438. [C
129. Dammeijer, F.; Lau, S.P.; van Eijck, C.H.J.; van d
to improve efficacy of immune checkpoint blockade in solid tumors. *Cytokine Growth Factor Rev.* **2017**, *36*, 5–15. [CrossRef]

http://dx.doi.org/10.1158/2326-6066.CIR-15

130. Kamada, T.; Togashi, Y.; Tay, C.; Ha, D.; Sasaki, A.; Nakamura, Y.; Sato, E.; Fukuoka, S.; Tada, Y.; Tanaka, A.; et al. PD-1^{+} regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 9999–10008. [CrossRef]
131. Liao, W.; Overman, M.J.; Boutin, A.T.; Shang, X.; Zhao, D.; Dey, P.; Li, J.; Wang, G.; Lan, Z.; Li, J.; et al. KRAS-IRF2 Axis Drives Immune Suppression and Immune Therapy Resistance in Colorectal Cancer. *Cancer Cell* **2019**, *35*, 559–572.e7. [CrossRef]
132. Dosset, M.; Vargas, T.R.; Lagrange, A.; Boidot, R.; Vegran, F.; Roussey, A.; Chalmin, F.; Dondaine, L.; Paul, C.; Lauret Marie-Joseph, E.; et al. PD-1/PD-L1 pathway: An adaptive immune resistance mechanism to immunogenic chemotherapy in colorectal cancer. *Oncoimmunology* **2018**, *7*, e1433981. [CrossRef]
133. Guan, Y.; Kraus, S.G.; Quaney, M.J.; Daniels, M.A.; Mitchem, J.B.; Teixeiro, E. FOLFOX Chemotherapy Ameliorates CD8 T Lymphocyte Exhaustion and Enhances Checkpoint Blockade Efficacy in Colorectal Cancer. *Front. Oncol.* **2020**, *10*, 586. [CrossRef] [PubMed]
134. Leon-Cabrera, S.A.; Molina-Guzman, E.; Delgado-Ramirez, Y.G.; Vazquez-Sandoval, A.; Ledesma-Soto, Y.; Perez-Plasencia, C.G.; Chirino, Y.I.; Delgado-Buenrostro, N.L.; Rodriguez-Sosa, M.; Vaca-Paniagua, F.; et al. Lack of STAT6 Attenuates Inflammation and Drives Protection against Early Steps of Colitis-Associated Colon Cancer. *Cancer Immunol. Res.* **2017**, *5*, 385–396. [CrossRef] [PubMed]
135. Mendoza-Rodriguez, M.G.; Sanchez-Barrera, C.A.; Callejas, B.E.; Garcia-Castillo, V.; Beristain-Terrazas, D.L.; Delgado-Buenrostro, N.L.; Chirino, Y.I.; Leon-Cabrera, S.A.; Rodriguez-Sosa, M.; Gutierrez-Cirlos, E.B.; et al. Use of STAT6 Phosphorylation Inhibitor and Trimethylglycine as New Adjuvant Therapies for 5-Fluorouracil in Colitis-Associated Tumorigenesis. *Int. J. Mol. Sci.* **2020**, *21*, 2130. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Referencias

1. Weiss, G. and U.E. Schaible, *Macrophage defense mechanisms against intracellular bacteria.* Immunological reviews, 2015. **264**(1): p. 182-203.
2. Koo, S.-j., et al., *Macrophages promote oxidative metabolism to drive nitric oxide generation in response to Trypanosoma cruzi.* Infection and immunity, 2016. **84**(12): p. 3527-3541.
3. Chávez-Galán, L., et al., *Much more than M1 and M2 macrophages, there are also CD169+ and TCR+ macrophages.* Frontiers in immunology, 2015. **6**: p. 263.
4. Rassi Jr, A., A. Rassi, and J.A. Marin-Neto, *Chagas disease.* The Lancet, 2010. **375**(9723): p. 1388-1402.
5. David Sibley, L., *Invasion and intracellular survival by protozoan parasites.* Immunological reviews, 2011. **240**(1): p. 72-91.
6. Fearon, M.A., et al., *A case of vertical transmission of Chagas disease contracted via blood transfusion in Canada.* Canadian Journal of Infectious Diseases and Medical Microbiology, 2013. **24**(1): p. 32-34.
7. Kransdorf, E.P., P.C. Zakowski, and J.A. Kobashigawa, *Chagas disease in solid organ and heart transplantation.* Current opinion in infectious diseases, 2014. **27**(5): p. 418-424.
8. Shikanai-Yasuda, M.A. and N.B. Carvalho, *Oral transmission of Chagas disease.* Clinical Infectious Diseases, 2012. **54**(6): p. 845-852.
9. Carlier, Y., et al., *Congenital Chagas disease: an update.* Memórias do Instituto Oswaldo Cruz, 2015. **110**(3): p. 363-368.
10. Abad-Franch, F., et al., *On palms, bugs, and Chagas disease in the Americas.* Acta tropica, 2015. **151**: p. 126-141.
11. de Souza, W., T.M.U. de Carvalho, and E.S. Barrias, *Review on Trypanosoma cruzi: host cell interaction.* International journal of cell biology, 2010. **2010**.
12. Salud, O.M.d.l., *La enfermedad de Chagas (tripanosomiasis americana).* Organización Mundial de la Salud, 2017.
13. Bern, C., et al., *Trypanosoma cruzi and Chagas' disease in the United States.* Clinical microbiology reviews, 2011. **24**(4): p. 655-681.
14. Angheben, A., et al., *Chagas disease in Italy: breaking an epidemiological silence.* Euro Surveill, 2011. **16**: p. 6-13.
15. Medina-Torres, I., et al., *Risk factors associated with triatomines and its infection with Trypanosoma cruzi in rural communities from the southern region of the state of Mexico, Mexico.* The American journal of tropical medicine and hygiene, 2010. **82**(1): p. 49-54.
16. Machado, F.S., et al., *Pathogenesis of Chagas disease: time to move on.* Frontiers in bioscience (Elite edition), 2012. **4**: p. 1743.

17. Flávia Nardy, A., C.G. Freire-de-Lima, and A. Morrot, *Immune evasion strategies of Trypanosoma cruzi.* Journal of immunology research, 2015. **2015**.

18. Castro, D.P., et al., *Trypanosoma cruzi: ultrastructural studies of adhesion, lysis and biofilm formation by Serratia marcescens.* Experimental parasitology, 2007. **117**(2): p. 201-207.

19. Alves, C.R., et al., *Trypanosoma cruzi: attachment to perimicrovillar membrane glycoproteins of Rhodnius prolixus.* Experimental parasitology, 2007. **116**(1): p. 44-52.

20. Nogueira, N.F., et al., *Trypanosoma cruzi: involvement of glycoinositolphospholipids in the attachment to the luminal midgut surface of Rhodnius prolixus.* Experimental parasitology, 2007. **116**(2): p. 120-128.

21. de Carvalho, T.M.U. and W. de Souza, *Early events related with the behaviour of Trypanosoma cruzi within an endocytic vacuole in mouse peritoneal macrophages.* Cell structure and function, 1989. **14**(4): p. 383-392.

22. Epting, C.L., B.M. Coates, and D.M. Engman, *Molecular mechanisms of host cell invasion by Trypanosoma cruzi.* Experimental parasitology, 2010. **126**(3): p. 283-291.

23. Almeida, I.C. and R.T. Gazzinelli, *Proinflammatory activity of glycosylphosphatidylinositol anchors derived from Trypanosoma cruzi: structural and functional analyses.* Journal of leukocyte biology, 2001. **70**(4): p. 467-477.

24. Ropert, C., et al., *Macrophage signaling by glycosylphosphatidylinositol-anchored mucin-like glycoproteins derived from Trypanosoma cruzi trypomastigotes.* Microbes and Infection, 2002. **4**(9): p. 1015-1025.

25. Aoki, M.P., et al., *Nonimmune cells contribute to crosstalk between immune cells and inflammatory mediators in the innate response to Trypanosoma cruzi infection.* Journal of parasitology research, 2012. **2012**.

26. Silva, J., et al., *Tumor necrosis factor alpha mediates resistance to in mice by inducing nitric oxide production in infected IFN-γ-activated macrophages.* Infect. Immun, 1995. **63**: p. 4862-4867.

27. Aliberti, J., et al., *IL-12 mediates resistance to Trypanozoma cruzi infection in mice and is produced by normal murine macrophages in response to live trypomastigote.* Infect. Immun, 1996. **64**: p. 1961-1967.

28. Van Voorhis, W.C., *Coculture of human peripheral blood mononuclear cells with Trypanosoma cruzi leads to proliferation of lymphocytes and cytokine production.* The Journal of Immunology, 1992. **148**(1): p. 239-248.

29. Ropert, C. and R.T. Gazzinelli, *Signaling of immune system cells by glycosylphosphatidylinositol (GPI) anchor and related structures derived from parasitic protozoa.* Current opinion in microbiology, 2000. **3**(4): p. 395-403.

30. Cardoso, M.S., J.L. Reis-Cunha, and D.C. Bartholomeu, *Evasion of the immune response by Trypanosoma cruzi during acute infection.* Frontiers in Immunology, 2016. **6**.

31. Xie, Z., M. Singh, and K. Singh, *ERK1/2 and JNKs, but not p38 kinase, are involved in reactive oxygen species-mediated induction of osteopontin gene expression by angiotensin II and interleukin-1β in adult rat cardiac fibroblasts.* Journal of cellular physiology, 2004. **198**(3): p. 399-407.

32. Deng, Y.T., H.C. Huang, and J.K. Lin, *Rotenone induces apoptosis in MCF-7 human breast cancer cell-mediated ROS through JNK and p38 signaling.* Molecular Carcinogenesis, 2010. **49**(2): p. 141-151.

33. Kibbe, M.R., et al., *Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate.* Journal of vascular surgery, 2000. **31**(6): p. 1214-1228.

34. Hannemann, N., et al., *The AP-1 Transcription Factor c-Jun Promotes Arthritis by Regulating Cyclooxygenase-2 and Arginase-1 Expression in Macrophages.* J Immunol, 2017. **198**(9): p. 3605-3614.

35. Dutra, W.O., et al., *Immunoregulatory networks in human Chagas disease.* Parasite immunology, 2014. **36**(8): p. 377-387.

36. Campos, M.A. and R.T. Gazzinelli, *Trypanosoma cruzi and its components as exogenous mediators of inflammation recognized through Toll-like receptors.* Mediators of inflammation, 2004. **13**(3): p. 139-143.

37. Tarleton, R.L., *Immune system recognition of Trypanosoma cruzi.* Current opinion in immunology, 2007. **19**(4): p. 430-434.

38. Kayama, H. and K. Takeda, *The innate immune response to Trypanosoma cruzi infection.* Microbes and infection, 2010. **12**(7): p. 511-517.

39. Jiang, D., et al., *Regulation of lung injury and repair by Toll-like receptors and hyaluronan.* Nature medicine, 2005. **11**(11): p. 1173.

40. Zhang, Z. and H. Schluesener, *Mammalian toll-like receptors: from endogenous ligands to tissue regeneration.* Cellular and Molecular Life Sciences CMLS, 2006. **63**(24): p. 2901-2907.

41. Li, C., et al., *Modulating Toll-like receptor mediated signaling by (1→ 3)-β-D-glucan rapidly induces cardioprotection.* Cardiovascular Research, 2004. **61**(3): p. 538-547.

42. Hua, F., et al., *Protection against myocardial ischemia/reperfusion injury in TLR4-deficient mice is mediated through a phosphoinositide 3-kinase-dependent mechanism.* The Journal of Immunology, 2007. **178**(11): p. 7317-7324.

43. Maganto-Garcia, E., et al., *Rab5 Activation by Toll-Like Receptor 2 is Required for Trypanosoma cruzi Internalization and Replication in Macrophages.* Traffic, 2008. **9**(8): p. 1299-1315.

44. Singh, S.K., et al., *Characterization of murine MGL1 and MGL2 C-type lectins: distinct glycan specificities and tumor binding properties.* Mol Immunol, 2009. **46**(6): p. 1240-9.

45. Oliveira, A.-C., et al., *Expression of functional TLR4 confers proinflammatory responsiveness to Trypanosoma cruzi glycoinositolphospholipids and higher resistance to infection with T. cruzi.* The Journal of Immunology, 2004. **173**(9): p. 5688-5696.

46. Pellegrini, A., et al., *The role of Toll-like receptors and adaptive immunity in the development of protective or pathological immune response triggered by the Trypanosoma cruzi protozoan.* Future microbiology, 2011. **6**(12): p. 1521-1533.

47. Kerrigan, A.M. and G.D. Brown, *C-type lectins and phagocytosis.* Immunobiology, 2009. **214**(7): p. 562-75.

48. Vajjhala, P.R., et al., *The molecular mechanisms of signaling by cooperative assembly formation in innate immunity pathways.* Molecular immunology, 2017. **86**: p. 23-37.

49. West, I. and O. Goldring, *Lectin affinity chromatography.* Protein Purification Protocols, 1996: p. 177-186.

50. Sancho, D. and C. Reis e Sousa, *Signaling by myeloid C-type lectin receptors in immunity and homeostasis.* Annual review of immunology, 2012. **30**: p. 491-529.

51. Dam, T.K. and C.F. Brewer, *Lectins as pattern recognition molecules: the effects of epitope density in innate immunity.* Glycobiology, 2009. **20**(3): p. 270-279.

52. Van Kooyk, Y., *C-type lectins on dendritic cells: key modulators for the induction of immune responses*. 2008, Portland Press Limited.

53. Duverger, E., et al., *Carbohydrate–lectin interactions assayed by SPR.* Surface Plasmon Resonance: Methods and Protocols, 2010: p. 157-178.

54. Vázquez-Mendoza, A., J.C. Carrero, and M. Rodriguez-Sosa, *Parasitic infections: a role for C-type lectins receptors.* BioMed research international, 2013. **2013**.

55. Chiffoleau, E., *C-Type Lectin-Like Receptors As Emerging Orchestrators of Sterile Inflammation Represent Potential Therapeutic Targets.* Frontiers in immunology, 2018. **9**: p. 227.

56. van Liempt, E., et al., *Schistosoma mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation.* Molecular immunology, 2007. **44**(10): p. 2605-2615.

57. Raes, G., et al., *Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation.* J Leukoc Biol, 2005. **77**(3): p. 321-7.

58. Kumamoto, Y., et al., *Identification of sialoadhesin as a dominant lymph node counter-receptor for mouse macrophage galactose-type C-type lectin 1.* J Biol Chem, 2004. **279**(47): p. 49274-80.

59. van Vliet, S.J., et al., *MGL-mediated internalization and antigen presentation by dendritic cells: a role for tyrosine-5.* Eur J Immunol, 2007. **37**(8): p. 2075-81.

60. Ropert, C., et al., *Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial lipopolysaccharide.* J Immunol, 2001. **166**(5): p. 3423-31.

61. van Vliet, S.J., E. Saeland, and Y. van Kooyk, *Sweet preferences of MGL: carbohydrate specificity and function.* Trends Immunol, 2008. **29**(2): p. 83-90.

62. Firestein, G.S., *A new murine CD4+ T cell subset with an unrestricted cytokine profile.* J. Immunol., 1989. **143**: p. 518-525.

63. van Vliet, S.J., et al., *MGL signaling augments TLR2-mediated responses for enhanced IL-10 and TNF-α secretion.* Journal of leukocyte biology, 2013. **94**(2): p. 315-323.

64. Vazquez, A., et al., *Mouse macrophage galactose-type lectin (mMGL) is critical for host resistance against Trypanosoma cruzi infection.* Int J Biol Sci, 2014. **10**(8): p. 909-20.

65. Dey, N., et al., *Caspase-1/ASC inflammasome-mediated activation of IL-1β–ROS–NF-κB pathway for control of Trypanosoma cruzi replication and survival is dispensable in NLRP3−/− macrophages.* PLoS One, 2014. **9**(11): p. e111539.

66. Tsuiji, M., et al., *Molecular cloning and characterization of a novel mouse macrophage C-type lectin, mMGL2, which has a distinct carbohydrate specificity from mMGL1.* Journal of Biological Chemistry, 2002. **277**(32): p. 28892-28901.

67. Muñoz, R. and W. Alfaro, *Estandarización de la técnica de reducción del NBT mediante lectura en placas de microELISA.* Revista Médica del Hospital Nacional de Niños Dr. Carlos Sáenz Herrera, 2000. **35**(1-2): p. 29-39.

68. Denda-Nagai, K., et al., *Distribution and Function of Macrophage Galactose-type C-type Lectin 2 (MGL2/CD301b) EFFICIENT UPTAKE AND PRESENTATION OF GLYCOSYLATED ANTIGENS BY DENDRITIC CELLS.* Journal of Biological Chemistry, 2010. **285**(25): p. 19193-19204.

69. Onami, T.M., et al., *Generation of mice deficient for macrophage galactose-and N-acetylgalactosamine-specific lectin: limited role in lymphoid and erythroid homeostasis and evidence for multiple lectins.* Molecular and cellular biology, 2002. **22**(14): p. 5173-5181.

70. Stahl, P., et al., *Trypanosoma cruzi evades the protective role of interferon-gamma-signaling in parasite-infected cells.* PLoS One, 2014. **9**(10): p. e110512.

71. Lykens, J.E., et al., *Mice with a selective impairment of IFN-gamma signaling in macrophage lineage cells demonstrate the critical role of IFN-gamma-activated macrophages for the control of protozoan parasitic infections in vivo.* J Immunol, 2010. **184**(2): p. 877-85.

72. Lykens, J.E., et al., *Mice with a Selective Impairment of IFN-γ Signaling in Macrophage Lineage Cells Demonstrate the Critical Role of IFN-γ–Activated Macrophages for the Control of Protozoan Parasitic Infections In Vivo.* The journal of immunology, 2009: p. ji_0902346.

73. Bogdan, C., *Nitric oxide and the immune response.* Nature immunology, 2001. **2**(10): p. 907-916.

74. Hibbs Jr, J.B., *Infection and nitric oxide.* The Journal of infectious diseases, 2002. **185**(Supplement_1): p. S9-S17.

75. Nathan, C. and M.U. Shiloh, *Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens.* Proceedings of the National Academy of Sciences, 2000. **97**(16): p. 8841-8848.

76. Machado-Silva, A., et al., *How Trypanosoma cruzi deals with oxidative stress: antioxidant defence and DNA repair pathways.* Mutation Research/Reviews in Mutation Research, 2016. **767**: p. 8-22.

77. Panis, C., et al., *Trypanosoma cruzi: effect of the absence of 5-lipoxygenase (5-LO)-derived leukotrienes on levels of cytokines, nitric oxide and iNOS expression in cardiac tissue in the acute phase of infection in mice.* Exp Parasitol, 2011. **127**(1): p. 58-65.

78. Rodrigues, A.A., et al., *IFN-gamma plays a unique role in protection against low virulent Trypanosoma cruzi strain.* PLoS Negl Trop Dis, 2012. **6**(4): p. e1598.

79. Campos, M.A., et al., *Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite.* J Immunol, 2001. **167**(1): p. 416-23.

80. Campos, M.A. and R.T. Gazzinelli, *Trypanosoma cruzi and its components as exogenous mediators of inflammation recognized through Toll-like receptors.* Mediators Inflamm, 2004. **13**(3): p. 139-43.

81. Rodrigues, M.M., A.C. Oliveira, and M. Bellio, *The Immune Response to Trypanosoma cruzi: Role of Toll-Like Receptors and Perspectives for Vaccine Development.* J Parasitol Res, 2012. **2012**: p. 507874.

82. Uno, K., et al., *Toll-like receptor (TLR) 2 induced through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells.* Am J Physiol Gastrointest Liver Physiol, 2007. **293**(5): p. G1004-12.

83. Zelensky, A.N. and J.E. Gready, *The C-type lectin-like domain superfamily.* FEBS J, 2005. **272**(24): p. 6179-217.

84. van Kooyk, Y., *C-type lectins on dendritic cells: key modulators for the induction of immune responses.* Biochem Soc Trans, 2008. **36**(Pt 6): p. 1478-81.

85. Gutierrez, F.R., et al., *The effects of nitric oxide on the immune system during Trypanosoma cruzi infection.* Mem Inst Oswaldo Cruz, 2009. **104 Suppl 1**: p. 236-45.

86. Holscher, C., et al., *Defective nitric oxide effector functions lead to extreme susceptibility of Trypanosoma cruzi-infected mice deficient in gamma interferon receptor or inducible nitric oxide synthase.* Infect Immun, 1998. **66**(3): p. 1208-15.

87. Montero-Barrera, D., et al., *The macrophage galactose-type lectin-1 (MGL1) recognizes Taenia crassiceps antigens, triggers intracellular signaling, and is critical for resistance to this infection.* Biomed Res Int, 2015. **2015**: p. 615865.

88. Geijtenbeek, T.B., et al., *Self-and nonself-recognition by C-type lectins on dendritic cells.* Annu. Rev. Immunol., 2004. **22**: p. 33-54.

89. Guo, W., et al., *Imperatorin attenuates LPS-induced inflammation by suppressing NF-κB and MAPKs activation in RAW 264.7 macrophages.* Inflammation, 2012. **35**(6): p. 1764-1772.

90. Hoving, J.C., G.J. Wilson, and G.D. Brown, *Signalling C-Type lectin receptors, microbial recognition and immunity.* Cellular microbiology, 2014. **16**(2): p. 185-194.

91. Arce, F., et al., *Selective activation of intracellular signalling pathways in dendritic cells for cancer immunotherapy.* Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 2012. **12**(1): p. 29-39.

92. Ganesan, L.P., et al., *FcγR-induced production of superoxide and inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways.* Blood, 2006. **108**(2): p. 718-725.

93. Sayan, M. and B.T. Mossman, *The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases.* Particle and fibre toxicology, 2015. **13**(1): p. 51.

94. Lawlor, K.E. and J.E. Vince, *Ambiguities in NLRP3 inflammasome regulation: is there a role for mitochondria?* Biochimica et Biophysica Acta (BBA)-General Subjects, 2014. **1840**(4): p. 1433-1440.

95. Gringhuis, S.I., et al., *Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk.* Nature immunology, 2009. **10**(2): p. 203-213.

96. Rogers, N.C., et al., *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins.* Immunity, 2005. **22**(4): p. 507-517.

97. Fujiwara, N. and K. Kobayashi, *Macrophages in inflammation.* Current Drug Targets-Inflammation & Allergy, 2005. **4**(3): p. 281-286.

98. Ng, W.C., et al., *The macrophage galactose-type lectin can function as an attachment and entry receptor for influenza virus.* Journal of virology, 2014. **88**(3): p. 1659-1672.

99. Kessler, R.L., et al., *Recently differentiated epimastigotes from Trypanosoma cruzi are infective to the mammalian host.* Molecular microbiology, 2017. **104**(5): p. 712-736.

100. Stahl, P., et al., *Trypomastigotes and amastigotes of Trypanosoma cruzi induce apoptosis and STAT3 activation in cardiomyocytes in vitro.* Apoptosis, 2013. **18**(6): p. 653-663.

101. Ley, V., et al., *Amastigotes of Trypanosoma cruzi sustain an infective cycle in mammalian cells.* Journal of Experimental Medicine, 1988. **168**(2): p. 649-659.

102. Procópio, D.O., et al., *Trypanosoma cruzi: effect of protein kinase inhibitors and cytoskeletal protein organization and expression on host cell invasion by amastigotes and metacyclic trypomastigotes.* Experimental Parasitology, 1998. **90**(1): p. 1-13.

103. Barbosa, H.S., *Why studies on invasion of host cell by Trypanosoma cruzi using stablished cell lines or primary cell cultures give conflicting results?* 1999.

104. Kipnis, T., V. Calich, and W.D. da Silva, *Active entry of bloodstream forms of Trypanosoma cruzi into macrophages.* Parasitology, 1979. **78**(1): p. 89-98.

105. Andrade, L.O. and N.W. Andrews, *The Trypanosoma cruzi–host-cell interplay: location, invasion, retention.* Nature Reviews Microbiology, 2005. **3**(10): p. 819.

106. Ley, V., et al., *Amastigotes of Trypanosoma cruzi sustain an infective cycle in mammalian cells.* J Exp Med, 1988. **168**(2): p. 649-59.

107. Procopio, D.O., et al., *Trypanosoma cruzi: effect of protein kinase inhibitors and cytoskeletal protein organization and expression on host cell invasion by amastigotes and metacyclic trypomastigotes.* Exp Parasitol, 1998. **90**(1): p. 1-13.

108. Bogdan, C., *Nitric oxide and the immune response.* Nat Immunol, 2001. **2**(10): p. 907-16.

109. Soares, R.P., et al., *Intraspecies variation in Trypanosoma cruzi GPI-mucins: biological activities and differential expression of α-galactosyl residues.* The American journal of tropical medicine and hygiene, 2012. **87**(1): p. 87-96.

110. Oliveira, A.-C., et al., *Impaired innate immunity in Tlr4−/− mice but preserved CD8+ T cell responses against Trypanosoma cruzi in Tlr4-, Tlr2-, Tlr9-or Myd88-deficient mice.* PLoS Pathog, 2010. **6**(4): p. e1000870.

111. Silva, R.R., et al., *Interferon-gamma promotes infection of astrocytes by Trypanosoma cruzi.* PloS one, 2015. **10**(2): p. e0118600.

112. Uno, K., et al., *Toll-like receptor (TLR) 2 induced through TLR4 signaling initiated by Helicobacter pylori cooperatively amplifies iNOS induction in gastric epithelial cells.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2007. **293**(5): p. G1004-G1012.

113. Campos, M.A., et al., *Impaired production of proinflammatory cytokines and host resistance to acute infection with Trypanosoma cruzi in mice lacking functional myeloid differentiation factor 88.* The Journal of Immunology, 2004. **172**(3): p. 1711-1718.

114. Timmermans, K., et al., *Blueprints of signaling interactions between pattern recognition receptors: implications for the design of vaccine adjuvants.* Clinical and Vaccine Immunology, 2013. **20**(3): p. 427-432.

115. Chan, E.D. and D.W. Riches, *IFN-γ+ LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 mapk in a mouse macrophage cell line.* American Journal of Physiology-Cell Physiology, 2001. **280**(3): p. C441-C450.

116. Schmidt, H., et al., *Regulation and subcellular location of nitrogen oxide synthases in RAW264. 7 macrophages.* Molecular pharmacology, 1992. **41**(4): p. 615-624.

117. Trinchieri, G. and A. Sher, *Cooperation of Toll-like receptor signals in innate immune defence.* Nature reviews. Immunology, 2007. **7**(3): p. 179.

118. Brown, G.D., J.A. Willment, and L. Whitehead, *C-type lectins in immunity and homeostasis.* Nature Reviews Immunology, 2018: p. 1.

119. Napoletano, C., et al., *Targeting of macrophage galactose-type C-type lectin (MGL) induces DC signaling and activation.* European journal of immunology, 2012. **42**(4): p. 936-945.

120. Núñez, R.D.P., *Regulación de la expresión del Receptor Tipo Toll 4 (TLR4) por el Factor de Necrosis Tumoral α y Glucocorticoides.*

121. Garrido, V.V., et al., *The increase in mannose receptor recycling favors arginase induction and Trypanosoma cruzi survival in macrophages.* International journal of biological sciences, 2011. **7**(9): p. 1257.

122. Ropert, C., et al., *Requirement of MAP kinases and IkB phosphorylation for induction of proinflammatory cytokines by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial LPS.* J Immunol, 2001. **166**: p. 3423.

123. Terrazas, C.A., et al., *Helminth-excreted/secreted products are recognized by multiple receptors on DCs to block the TLR response and bias Th2 polarization in a cRAF dependent pathway.* The FASEB Journal, 2013. **27**(11): p. 4547-4560.

124. Shaul, Y.D. and R. Seger, *The MEK/ERK cascade: from signaling specificity to diverse functions.* Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2007. **1773**(8): p. 1213-1226.

125. Chang, F., et al., *Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention.* Leukemia (08876924), 2003. **17**(7).

126. Jung, D.-H., et al., *Diosgenin inhibits macrophage-derived inflammatory mediators through downregulation of CK2, JNK, NF-κB and AP-1 activation.* International immunopharmacology, 2010. **10**(9): p. 1047-1054.

127. Bae, H.-B., et al., *Sauchinone, a lignan from Saururus chinensis, reduces tumor necrosis factor-α production through the inhibition of c-raf/MEK1/2/ERK 1/2 pathway activation.* International immunopharmacology, 2010. **10**(9): p. 1022-1028.

128. Korhonen, R., et al., *Nitric oxide production and signaling in inflammation.* Current Drug Targets-Inflammation & Allergy, 2005. **4**(4): p. 471-479.

129. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-820.

130. van Vliet, S.J., et al., *MGL-mediated internalization and antigen presentation by dendritic cells: A role for tyrosine-5.* European journal of immunology, 2007. **37**(8): p. 2075-2081.

131. Nunes, M.P., et al., *Inhibitory effects of Trypanosoma cruzi sialoglycoproteins on CD4+ T cells are associated with increased susceptibility to infection.* PloS one, 2013. **8**(10): p. e77568.

132. Medeiros, M.M., et al., *Toll-like receptor 4 (TLR4)-dependent proinflammatory and immunomodulatory properties of the glycoinositolphospholipid (GIPL) from Trypanosoma cruzi.* Journal of leukocyte biology, 2007. **82**(3): p. 488-496.

133. Chamekh, M., et al., *Transfection of Trypanosoma cruzi with host CD40 ligand results in improved control of parasite infection.* Infection and immunity, 2005. **73**(10): p. 6552-6561.

134. Gonçalves, V.M., et al., *NLRP3 controls Trypanosoma cruzi infection through a caspase-1-dependent IL-1R-independent NO production.* PLoS neglected tropical diseases, 2013. **7**(10): p. e2469.

135. Geijtenbeek, T.B. and S.I. Gringhuis, *C-type lectin receptors in the control of T helper cell differentiation.* Nature Reviews Immunology, 2016. **16**(7): p. 433.