UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

PAPEL DE LAS MOLÉCULAS DE STAT1 Y STAT6 EN LA SUSCEPTIBILIDAD Y PATOLOGÍA PULMONAR DURANTE LA INFECCIÓN POR *Toxocara canis*

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTORA EN CIENCIAS

PRESENTA:
BERENICE FAZ LÓPEZ

DIRECTOR DE TESIS
DR. LUIS IGNACIO TERRAZAS VALDÉZ
FACULTAD DE ESTUDIOS SUPERIORES IZTACALA

COMITÉ TUTOR
DR. JULIO CÉSAR CARRERO SANCHEZ
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS
DR. ABRAHAM LANDA PIEDRA
FACULTAD DE MEDICINA

LOS REYES IZTACALA, ESTADO DE MÉXICO. FEBRERO DE 2020

AGRADECIMIENTOS

A la UNAM quien me dio un lugar en su casa y siempre me ha cobijado en sus recintos para poderme desarrollar profesionalmente

Al Programa de Doctorado en Ciencias Biomédicas (PDCB) por permitirme ser parte de este programa de doctorado.

A Consejo Nacional de Ciencia y Teccnología (CONACyT) por el apoyo económico otorgado (515378).

Al M. en C. Juan Pablo Martínez Labat, a quien le debo que este proyecto se pudiera llevar a cabo gracias a sus enseñanzas y colaboración.

A mi tutor el Dr. Luis Ignacio Terrazas Valdés quien me tuvo una infinita paciencia y me apoyo no sólo en el aspecto académico del proyecto sino personal para poder concluir esta etapa tan importante de mi vida, a quien he admirado desde que lo conozco y siempre será una parte importante de mi carrera profesional.

DEDICATORIAS

A mi papá quien me enseñó a ser fuerte e independiente, siempre me impulso a seguir estudiando, del cual aprendí muchos valores y virtudes y le estaré agradecida de por vida por todo lo que ha hecho por mí.

A mi mamá de quien aprendí a ser bondadosa y humilde, quien siempre ha creído en mí, ha estado para darme ánimos de seguir adelante y reconfortarme cuando más lo he necesitado.

A mis hermanas, con las cuales he crecido no sólo como familia, sino como persona y profesionalmente.

A mi amado, quien me ha apoyado en este proceso de manera incondicional y quien mejor que nadie entiende el esfuerzo que este trabajo representa.

A Angelina Reyes Harris quien dio vida a una de las personas más importantes de mi vida, que siempre he admirado por su gran valor, entereza y coraje y por su apoyo incondicional.

A mis niñas que a pesar de ser bebés, me han enseñado que debo seguir adelante ya que son una razón más para no detenerme en esta carrera que apenas empieza, y que los obstáculos que existen en la vida sólo son mentales.

A mis amigos que no sólo me apoyaron en la elaboración de este trabajo, sino que además estuvieron en momentos difíciles de mi vida.

A todos muchas gracias por su tiempo, paciencia, cariño, amor y comprensión.

INDICE

ABREVIATURAS

Arg - Arginasa
CD – Clúster de diferenciación
ChiL o Ym – Quitinasas
CT – Larva Cutánea
Hp - *Heligmosomoides polygyrus*
IFN – Interferón
Ig – Inmunoglobulina
IL - Interleucina
ILCs – Linfocitos de la inmunidad innata
IL-4Rα - receptor alfa de IL-4
iNOS – Óxido Nítrico Sintasa inducible
IRF – Factor Regulador del Interferón
IRF – Factores Reguladores de Interferón
JAKs – Janus Cinasas TNF – Factor de Necrosis Tumoral
LMO - Larva Migrans Ocular
LMV - Larva Migrans Visceral
L2 – Larva 2
MMP – Metaloproteasas de matriz
MMR – Receptor de manosa murino
M1 – Macrófagos clásicamente activados o Inflamatorios
M2 – Macrófagos alternativamente activados o antiinflamatorios
NADPH - Dinucleotido adenin-nicotinamida
NT – Neurotoxocariosis
ON – Óxido Nítrico
Retnl, Relm o FIZZ - moléculas tipo resistina
ROS - Especies Reactivas de Oxigeno
STAT – Transductor de Señalización y Activador de transcripción
T. canis – *Toxocara canis*
TGF - Factor de Crecimiento Tumoral
Th1 – Células T cooperadoras tipo-1
Th2 – Células T cooperadoras tipo-2
$STAT1^{-/-}$ – Ratones deficientes de STAT1
$STAT6^{-/-}$ – Ratones deficientes de STAT6
WT – Wild Type (Ratones Silvestres)

RESUMEN

Los macrófagos clásicamente activados (M1) a través de la vía de señalización IFN-γ/ STAT1, tienen un importante papel en la mediación de la inflamación durante infecciones microbianas y parasitarias. En algunos casos, la desregulación de dicha inflamación conlleva al desarrollo de daño tisular. En diversas infecciones por helmintos, los macrófagos alternativamente activados (M2), mediante la vía de señalización IL-4/STAT6, tienen un papel protector contra la inflamación excesiva, lo cual se ha asociado tanto a la regeneración tisular como a la neutralización parasitaria. Durante las fases migratorias de *Toxocara canis* (*T. canis*), el papel de los macrófagos M1 y M2 en la regeneración tisular, es aún desconocido. Para tratar de entender dicho proceso, se infectaron vía oral ratones wild-type (WT) y STAT1 y STAT6 deficientes ($STAT1^{-/-}$ y $STAT6^{-/-}$) con L2 *T. canis*, y se evaluó el papel de los macrófagos en la patología pulmonar. La ausencia de STAT1 favoreció un patrón de activación alternativa caracterizado por la expresión de Arg1, FIZZ1 y YM1, asociado con la resistencia parasitaria y regeneración tisular pulmonar. En contraste, la ausencia de STAT6 indujo la activación de M1 y producción de iNOS, lo cual ayudo al control de la infección parasitaria, pero en su lugar indujo mayor inflamación, asociada con la patología pulmonar. Para determinar la importancia de los macrófagos en dicho fenómeno, fueron eliminados a través de la inoculación intratraqueal (it) de liposomas con PBS o liposomas con clodronato en las tres cepas de ratones, antes de ser infectados con L2 *T. canis.* De dicho experimento, se encontró una reducción significativa de los macrófagos alveolares, que fue asociada con una mayor patología pulmonar en ratones WT y $STAT1^{-/-}$, mientras que los ratones $STAT6^{-/-}$ que recibieron liposomas con clodronato, mostraron menor daño tisular, indicando que ambos fenotipos celulares de macrófagos tienen papeles críticos en la patología y reparación del tejido pulmonar. A sí mismo, un balance apropiado entre una respuesta inflamatoria y anti-inflamatoria durante la infección por *T. canis*, es necesaria para limitar la patología pulmonar y favorecer la regeneración pulmonar.

Palabras clave: STAT1; STAT6; macrófagos M1-M2; daño-reparación tisular; resistencia parasitaria

ABSTRACT

Macrophages that are classically activated (M1) through the IFN-γ/STAT1 signaling pathway have a major role in mediating inflammation during microbial and parasitic infections. In some cases, unregulated inflammation induces tissue damage. In many helminth infections, alternatively activated macrophages (M2), whose activation is mainly mediated via the IL-4/STAT6 pathway, have a significant role in mediating protection against excessive inflammation, which has been associated with both tissue repair and parasite clearance. During the migratory lung stage of *Toxocara canis* infection, the roles of M1 and M2 macrophages in tissue repair remain unknown. To achieve this aim, we orally infected wild-type (WT) and STAT1 and STAT6-deficient mice ($STAT1^{-/-}$ and $STAT6^{-/-}$) with L2 *T. canis*, and the role of M1 or M2 macrophages in lung pathology was evaluated. The absence of STAT1 favored an M2 activation pattern with Arg1, FIZZ1 and Ym1 expression, which resulted in parasite resistance and lung tissue repair. In contrast, the absence of STAT6 induced M1 activation and iNOS production, which helped to control parasitic infection but generated increased inflammation associated with lung pathology. To determine the involvement of macrophages in these phenomena, macrophages were depleted by intratracheally inoculating mice with clodronate-loaded liposomes before challenge with L2 *T. canis*. We found a significant reduction in alveolar macrophages that was associated with higher lung pathology in both WT and $STAT1^{-/-}$ mice, whereas $STAT6^{-/-}$ mice receiving clodronate-liposomes displayed less tissue damage, indicating critical roles of both macrophage phenotypes in lung pathology and tissue repair. Therefore, a proper balance between both the inflammatory response and anti-inflammatory response during *T. canis* infection is necessary to limit lung pathology and favor lung healing.

Keywords: STAT1; STAT6; M1-M2 macrophages; tissue damage-repair; parasite resistance

INTRODUCCIÓN

La toxocariasis es una enfermedad olvidada a pesar de estar ampliamente distribuida, a la cual se ha prestado poca atención en materia de prevención, tratamiento y vigilancia epidemiológica [1]. Los gusanos adultos de *Toxocara canis* (*T. canis*) viven en el intestino delgado de los perros, que excretan los huevos en las heces, contaminando el medio ambiente [2]. Una vez en el intestino delgado de los huéspedes intermedios (pueden ser huéspedes paraténicos, como humanos o ratones), las larvas penetran en la pared intestinal y migran a diversos órganos, incluidos pulmones, hígado, músculos y sistema nervioso central, dando como resultado el desarrollo de inflamación y daño tisular. La infección en el pulmón con larvas de *T. canis* puede causar una afección de tipo asmático en niños [3, 4]; induciendo hiperreactividad de vías respiratorias, inflamación pulmonar y el aumento sistémico en los niveles de Inmunoglobulina (Ig)E; mismos signos que pueden persistir durante meses en ratones infectados experimentalmente con *T. canis* [5, 6]. Por lo tanto, evaluar la respuesta inflamatoria por parte de células como los macrófagos en la patología después de la infección con *T. canis*, es fundamental para una mejor comprensión de la enfermedad y el desarrollo de terapias dirigidas.

Los macrófagos M1 se activan principalmente mediante el interferón (IFN) -γ a través de vías de señalización como el transductor de señal y el activador de la transcripción 1 (STAT1), y los factores reguladores de interferón (IRF) 1 y 8, que permiten la expansión de la respuesta inmune inflamatoria [7, 8]. Producen citocinas inflamatorias como el Factor de Necrosis Tumoral (TNF)-α, Interleucina (IL)-1β, IL-6, IL-12 e IL-23 y óxido nítrico (ON) a través de la enzima óxido nítrico sintasa inducible (iNOS) [9], y presentan una marcada expresión de moléculas co-estimuladoras CD80 y CD86 [10]. Por otro lado, durante la infección por helmintos, existen potentes redes antiinflamatorias e inmuno-reguladoras que pueden limitar el daño tisular causado por el parásito (o la respuesta inmune contra ellos). Una característica distintiva celular de la inmunidad antiparasitaria son los macrófagos activados de manera alternativa (M2) por la IL-4 e IL-13 a través del receptor alfa de IL-4 (IL-4Rα) y vía STAT6, c- Myc e IRF4 [7, 11-13]. Por lo tanto, la activación de M2 promueve el control a la infección por helmintos y la reparación de tejidos [14, 15]. STAT6 regula muchos de los genes asociados con los macrófagos M2 de ratón, incluida la arginasa 1 (Arg1), moléculas tipo resistina-α (Retnl- α, Relm α o FIZZ1), la quitinasa 3-tipo 3 (Chi3L3 o Ym1) y el receptor de manosa (MMR) o CD206. Los macrófagos M2 tienen la capacidad de bloquear iNOS, contrarrestando el daño tisular causado por los macrófagos M1 [8, 10]. Además, los macrófagos M2 activados a través de STAT6

son una fuente importante de quimiocinas, citocinas, elastina, metaloproteinasas de matriz (MMP), Factor de Crecimiento Tumoral (TGF)-β y otros mediadores que impulsan las respuestas celulares después de una lesión tisular [16, 17].

Varios estudios en ratones infectados con nematodos demuestran la relevancia del equilibrio entre los macrófagos M1 y M2 y las moléculas que liberan estas células [18]. En particular, los estudios que involucran *Heligmosomoides polygyrus* [19] y *Nippostrongylus brasiliensis* [20], así como los nematodos filariales *Brugia malayi* y *Litomosoides sigmodontis* [21], y el trematodo *Schistosoma japonicum* [22] destacan una respuesta inflamatoria inicial de tipo T cooperadora-1 (Th1)-M1 asociada con daño tisular, seguido de una polarización Th2-M2 que promueve la reparación del tejido dañado. Está claro que los macrófagos participan en muchas funciones inmunes importantes después de la infección con helmintos que se caracterizan por la expresión de marcadores exclusivos del perfil M2 (por ejemplo, Arg1, FIZZ1 e Ym1) [23]. Sin embargo, existen datos limitados sobre la implicación de la activación de macrófagos a través de STAT1 y STAT6 durante la segunda etapa infecciosa de larvas (L2) de *T. canis*. Previamente, informamos que las citocinas Th2 tienen un doble papel durante la toxocariasis. Por un lado, la activación de STAT6 contribuye a la susceptibilidad del huésped y, alternativamente, la regulación de la respuesta inmune inflamatoria reduce la inmunopatología inducida por la infección por *T. canis* en los pulmones [24].

A pesar de estos hallazgos, aún quedan preguntas sin resolver. No se ha determinado si los macrófagos M2 son las principales células involucradas en la reparación de tejidos mediante su activación por STAT6. Además, el papel de los macrófagos M1 después de la infección por L2 de *T. canis* sigue siendo desconocido. Para comprender mejor las posibles funciones desempeñadas por los macrófagos M1 y M2 durante la toxocariasis aguda, utilizamos ratones deficientes en STAT1 o STAT6. Después de la infección por L2 de *T. canis*, los ratones $STAT1^{-/-}$ favorecieron la activación de M2 determinada por el aumento en la expresión pulmonar de Arg1, FIZZ1 e Ym1, lo que resultó en resistencia al parásito y la reparación del tejido pulmonar. Los ratones $STAT6^{-/-}$ mostraron evidencia de activación M1 y expresión de iNOS, lo que ayudó a reducir la cantidad de larvas en diferentes tejidos, pero generó un aumento de la inflamación y una extensa patología pulmonar. Por lo tanto, los macrófagos M1 y M2 están involucrados en la patología asociada a *T. canis*. Sugerimos que durante la etapa migratoria pulmonar de *T. canis* es necesario un equilibrio en un momento específico entre la respuesta inflamatoria y antiinflamatoria para la protección contra *T. canis*.

1. ANTECEDENTES

1.1. Toxocara canis

T. canis es un parásito helminto perteneciente al *phyllum* Nematoda, el cual se caracteriza por ser multicelular y tener forma redondeada [25]. Es el principal agente etiológico causante de la toxocariosis, una de las zoonosis más comúnmente reportadas en infecciones por helmintos a nivel mundial, con una alta prevalencia en áreas tropicales y poblaciones rurales [2]. Algunos factores de riesgo asociados con la infección por este parásito son la edad, estatus socioeconómico, ingestión de carne cruda, pobre higiene, lavado de manos inadecuado y contacto con suelo contaminado con los huevecillos [26]. Los niños son el grupo social con mayor riesgo debido sus actividades de recreación, higiene y relaciones cercanas con mascotas [27]. Las especies más importantes distribuidas a nivel mundial y principales causantes de dicha zoonosis son los cánidos; perros, zorros, lobos, chacales y coyotes; ya que al ser los hospederos definitivos, excretan huevos de *T. canis* no embrionados a través de las heces, contaminando el medio ambiente [2]. Bajo condiciones favorables de temperatura y humedad, los huevos pasan a ser embrionados en cuestión de semanas o meses, los cuales albergan al segundo estadio infectivo del parásito (L2), y pueden permanecer en estado infectivo en el medio ambiente por meses e incluso años [28].

L2 de *T. canis* es, a menudo, ingerida de manera accidental por los humanos a través de comida, agua o del suelo contaminados. En el intestino delgado de sus hospederos la L2 emerge del huevecillo y migra a través de las paredes intestinales vía circulación sistémica a diferentes órganos; los cuales incluyen hígado, pulmón, sistema nervioso central y músculo, activando respuestas inmunes e inflamatorias que conllevan a la presentación de síntomas como fiebre, tos, dolores de cabeza, abdominal y de extremidades. Los síndromes clínicos asociados con la toxocariosis humana son: larva migrans visceral (LMV), larva migrans ocular (LMO), neurotoxocariosis (NT) y larva cutánea (CT). Los cuales son dependientes del sitio de migración del parásito y se relacionan en gran medida con su ciclo biológico [29, 30].

1.2. Ciclo biológico.

1.2.1. Toxocariosis en el huésped definitivo

Las formas adultas de *T. canis* (Figura 1a) viven en el tracto digestivo superior de sus hospederos definitivos caninos cachorros. Las hembras gusanos pueden producir arriba de 200,000 huevos por día, dichos huevos son eliminados a través de las heces como huevos inmaduros y requieren de un

período de incubación en el suelo para ser embrionados [31]. La embrionación ocurre después de una semana de su deposición en el suelo, sin embargo los períodos de incubación pueden variar dependiendo de las condiciones medio ambientales, ya que en climas más cálidos y húmedos el proceso es acelerado, mientras que en climas fríos el período de incubación es más largo. Los huevecillos embrionados de *T. canis* contienen el estadio infectivo L2 (Figura 1b), los cuales pueden permanecer en el medio ambiente por meses, e incluso años [32]. Cuando un perro ingiere los huevecillos embrionados, los huevos eclosionan en el intestino delgado, donde las larvas liberadas perforan la pared intestinal, mecanismo por el cual acceden a la circulación sanguínea y van a través del hígado y pulmón al ventrículo izquierdo cardíaco, donde son diseminadas a todo el organismo vía circulación sistémica. Las larvas de *T. canis* se pueden desarrollar a gusanos adultos en tres semanas y liberar huevecillo en el intestino de sus hospederos para ser excretados por medio de las heces [33].

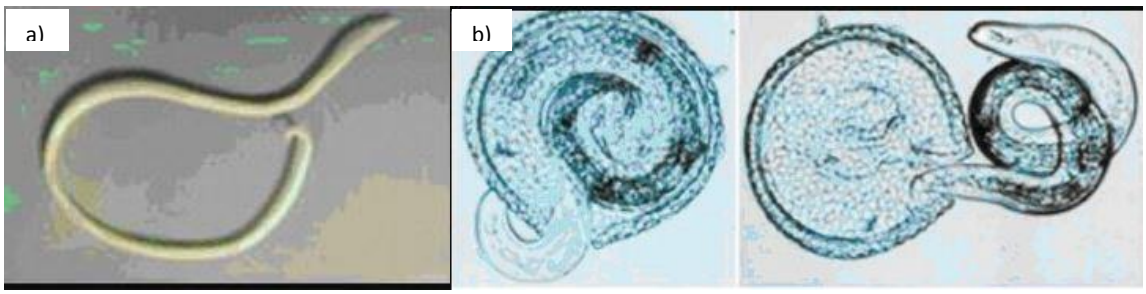

Figura 1. Estadio adulto de *Toxocara canis* (a). Estadio infectivo L2 de *T. canis* eclosionando de su huevecillo (b). Imagen tomada de Alessandra Nicoletti, 2013 [34].

En hembras caninas preñadas las larvas por estímulos hormonales, que han vivido en estado de hipobiosis, se activan y migran a través de la placenta infectando a los fetos. De hecho, se ha observado que la mayoría de los caninos cachorros, son infectados vía trans-placentaria o trans-mamaria, los cuales resultan ser los principales diseminadores de huevecillos al medio ambiente hasta los 24 meses de edad. Una vez que dichos cachorros alcanzan la etapa adulta dejan de ser hospederos definitivos y pasan a ser hospederos paraténicos. No se sabe con exactitud cuáles son los mecanismos involucrados de dicho fenómeno, pero se piensa que es debido a que en los cachorros el sistema inmunológico no se encuentra bien desarrollado, de tal forma que la larva puede evadirlo de forma eficiente para poder completar su ciclo biológico, cosa que en caninos adultos no es posible [35].

1.2.2. Toxocariosis en humanos y otras especies animales no caninas

Otras especies animales, incluyendo al humano, son considerados hospederos accidentales, ya que sólo sirven como medio de transporte hasta que el parásito encuentra a su hospedero definitivo para poder completar su ciclo. Una vez que los huevecillos son ingeridos de manera accidental, las L2 eclosionan en intestino delgado, pero dichos estadios presentan deficiencias para poder completar su desarrollo al estadio adulto. En su lugar, se mantienen migrando primero por hígado, luego pulmones y de igual forma acceden a la circulación sistémica hasta llegar a cerebro y músculo, siendo estos dos últimos los sitios en los que primordialmente las larvas entran en estado de hipobiosis manteniéndose durante meses e incluso años hasta encontrar de nuevo a su hospedero definitivo (Fig. 2). Diversos modelos murinos han sido utilizados con la finalidad de reproducir la infección y consecuentemente poder tener un mejor entendimiento del proceso de migración en los hospederos accidentales.

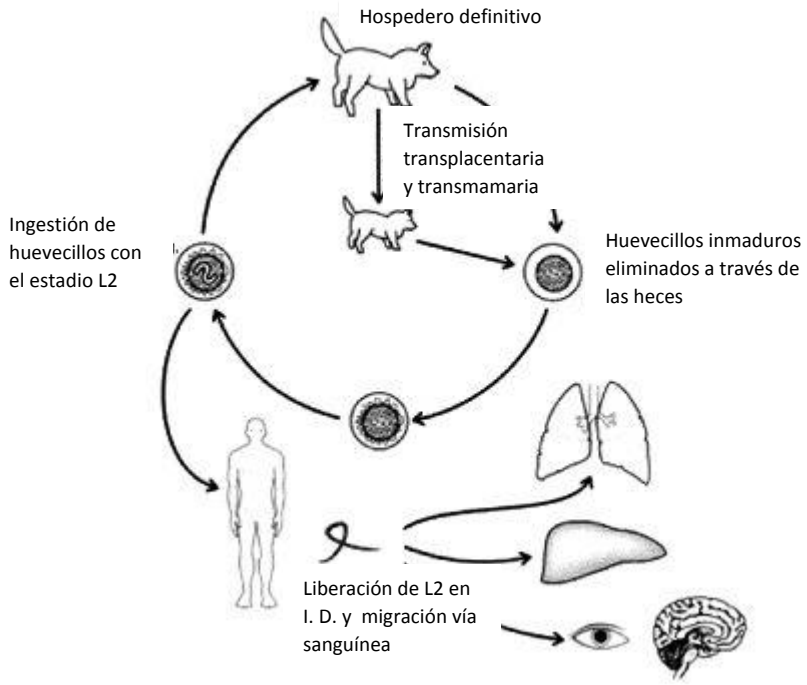

Figura 2. Ciclo de *Toxocara canis*. Después de que los huevecillos larvados son ingeridos por su hospedero definitivo, estos maduran en intestino delgado hasta estadio L4 con capacidad para reproducirse y dar origen a los huevecillos inmaduros que son eliminados al medio ambiente a través de las excretas y bajo condiciones óptimas de temperatura y humedad logran formar el estadio L2. Hospederos accidentales (humanos, roedores y otras especies no caninas) y definitivos los ingieren de manera accidental, las L2 eclosionan de su huevecillo en intestino delgado y migran por torrente sanguíneo a diferentes órganos (hígado, pulmón, cerebro, músculo, ojo) en donde pueden permanecer en modo latente hasta encontrar de nuevo a su hospedero definitivo (hospederos accidentales). O bien, hasta completar de nuevo la etapa adulta (hospederos definitivos). Hembras caninas también pueden transmitir los parásitos a sus crías vía trans-placentaria o trans-mamaria. Imagen modificada de Alessandra Nicoletti, 2013 [34].

1.3. Presentaciones clínicas

1.3.1. Larva Migrans Visceral

LMV es una enfermedad zoonótica causada por la migración del segundo estadio larvario del nematodo de *T. canis* a través de diversos órganos, siendo la población infantil, con historial de geofagia y convivencia cercana con cachorros en casa, la más comúnmente afectada por esta presentación. Los signos agudos asociados con la migración larvaria hepática y pulmonar a menudo incluyen dolor abdominal, disminución del apetito, fiebre, tos, estornudo, broncoespasmos parecidos al asma y hepatomegalia. En la fase de infección, usualmente hay una marcada eosinofília, leucocitosis e hipergamaglobulinemia de las clases IgM, IgE e IgG. De manera más infrecuente, miocarditis, nefritis y el compromiso de SNC, han sido también descritos [36, 37].

1.3.2. Larva Migrans Ocular.

LMO ocurre típicamente de manera unilateral en niños y adultos jóvenes. El síntoma más común es pérdida de la visión. La examinación oftalmológica revela uveítis, endoftalmitits, papilitis, lesión granulomatosa retinal o masas inflamatorias en la periferia del vítreo. La infección ocular también puede ser subclínica y sólo ser detectada durante examinaciones oftalmológicas de rutina [38, 39].

1.3.3. Neurotoxocariasis

Las presentaciones clínicas del sistema nervioso en LMV son raramente reportadas, a pesar de que en modelos murinos de experimentación, las larvas frecuentemente migran al cerebro y a pesar que LMV es visto de forma frecuente en niños, NT comúnmente reportada en adultos. Esta presentación se caracteriza por la manifestación de meningoencefalitis eosinofílica, meningitis, meningomielitis, meningoencefalomielitis, encefalitis y desordenes del comportamiento. También se han asociados cuadros epilépticos a la toxocariasis [40].

1.4. Prevalencia e incidencia de la toxocariasis.

T. canis es un parásito que se encuentra distribuido a nivel mundial, cuya incidencia es mayor en países subdesarrollados y en áreas rurales tanto de países desarrollados como subdesarrollados, debido a las pobres condiciones de higiene y acceso al agua potable que estas áreas en general presentan. Se ha asociado mayormente a países subdesarrollados debido al clima tropical prevalente en estos países, ya que los huevos del parásito tienen mayor probabilidad de madurar y sobrevivir a las condiciones medioambientales propias de climas tropicales. La primera infección humana fue reportada en 1950 y desde entonces ha sido reportada en al menos 100 países, con la

mayoría de casos reportados en Francia, Austria, India, Japón, Korea, China, Estados Unidos (E.U.) y Brasil [26].

En un meta análisis llevado a cabo para recopilar los datos de incidencia de *T. canis* en países de Norteamérica como Canadá, Estados Unidos y México; se encontraron 18 artículos referentes a seroprevalencia, incidencia o reporte de casos para toxocariasis; de los cuales 5 reportes fueron llevados a cabo en Canadá, 8 en Estados Unidos y otros 5 en México. El rango de prevalencia estimada fue de 0.6% en comunidades Inuit de Canadá, y hasta un 30.8% en niños mexicanos con asma. El rango de prevalencia estimada para Estados Unidos fue de 8.6% en niños de 1 a 5 años, hasta 15.1% en niños de 6 a 11 años, y un rango de prevalencia de 13.9% reportada para adultos y niños con edades mayores a 6 años. Un estimado de seroprevalencia reportado en México tiende a ser más alto que aquellos reportados en E.U. y Canadá; y los datos disponibles están enfocados en comparar los rangos de prevalencia en grupos de alto riesgo como pacientes psiquiátricos, recicladores de basura y niños asmáticos. La seroprevalencia de estos grupos de alto riesgo presentó un rango de 4.7 en pacientes psiquiátricos hospitalizados y hasta un 30.8 en niños con asma [41]

En México se han llevado a cabo estudios para determinar la prevalencia de *T. canis* en áreas recreacionales; y muestras de suelos y heces de perros han sido evaluados obteniendo rangos que van desde un 24% hasta un 67.5% del total de las muestras obtenidas. El alto rango de contaminación también se ha visto reflejado en el estatus socioeconómico y nivel de sanitización del área de estudio [42].

A sí mismo estudios de seroprevalencia en niños han reportado rangos de 22.22% en México y estudios realizados para detectar respuesta inmune en modelos murinos infectados previamente con larvas de *T. canis* han revelado la activación de células Th2, respuesta encargada de activar todos los procesos inmunológicos asociados con la reparación tisular en donde las citocinas IL-4, e IL-10 propios de una respuesta inmune antiinflamatoria se observan incrementadas tanto en suero como en lavados de tejido pulmonar, así como la activación de células B productoras de anticuerpos IgG1 e IgE. A su vez también se ha observado que las infecciones por este nematodo se caracterizan por generar una marcada eosinofília; todo ello en respuesta a la presencia del parásito [5, 42-44].

1.5. Respuesta inmune durante la infección por T. canis.

La infección por helmintos induce una respuesta inmune Th2, típicamente reflejada en la secreción de IL-4, IL-5 e IL-13, citocinas que están involucradas en la activación de mastocitos, eosinófilos y macrófagos; así como la producción de altos niveles de IgE. La activación de la respuesta Th2

normalmente coincide con la modulación descendente de la respuesta inflamatoria Th1, reduciendo la expresión de TNF-α, IFN-γ e IL-17 [45, 46]. Sin embargo, normalmente el sistema inmune organiza un cuidadoso equilibrio entre las respuestas pro y antiinflamatorias para funcionar eficientemente contra la infección por *T. canis*, aunque la supervivencia crónica de las larvas en huéspedes paraténicos indica que esto no siempre puede tener éxito en la erradicación de todos los gusanos.

En los hospedadores paraténicos, como los humanos y los ratones, las larvas de *T. canis* no se desarrollan hasta la etapa adulta, sino que migran a través del tejido somático y persisten como L2 durante largos períodos. En este contexto, la infección experimental en ratones, que imita la biología de la infección humana, podría ser relevante para una mejor comprensión de la toxocariasis humana [47-49]. Infecciones experimentales con *T. canis* en ratones revelaron eosinofília elevada con altos títulos de IgE después de la infección [44]. Además, los altos niveles plasmáticos de IL-6 e IFN-γ se correlacionaron con lesiones pulmonares [50], y en las infecciones crónicas por *T. canis*, se informó un predominio de la respuesta inmune Th2 caracterizado por un incremento en la producción de citocinas como IL-4, IL-13, IL-5 e IL-10 [51-53] respuesta que es la encargada de la activación de macrófagos alternativos [17, 54, 55].

1.6. Los macrófagos

Los macrófagos se originan de los monocitos circulantes, los cuales durante procesos de homeostasis e inflamación dejan el torrente sanguíneo para migrar hacía los tejidos donde se diferencian a macrófagos debido a su exposición a factores de crecimiento locales, citocinas del microambiente y productos anti-microbianos [56, 57]. Los macrófagos tienen múltiples funciones como fagocitosis, presentación antigénica, y la producción de diferentes citocinas [58]. Son esenciales para el control de enfermedades infecciosas, remoción de debridados y células muertas, promoviendo la regeneración tisular y la cicatrización, además contribuyen a la patología y daño tisular durante enfermedades inflamatorias e infecciosas [59, 60].

Los macrófagos son células con mucha plasticidad que pueden cambiar de un fenotipo a otro, cuya polarización se lleva a cabo a través de un cambio de fenotipo y una respuesta funcional específicos de acuerdo al estímulo micro-ambiental y de señalización [61]. Dos principales sub-poblaciones de macrófagos con diferentes funciones son los macrófagos clásicamente activados o inflamatorios (M1) y los macrófagos alternativamente activados o anti-inflamatorios (M2). Este fenómeno de dos diferentes fenotipos (M1/M2) es referido con el término “polarización de los macrófagos” [62-64].

1.6.1. Macrófagos M1

Los macrófagos M1 son típicamente inducidos por citocinas tipo Th1 como IFN-γ a través de la vía STAT-1. Una vez activados pueden producir y secretar altos niveles de citocinas inflamatorias como TNF-α, IL-1α, IL-1β, IL-6, IL-12 e IL-23 y tienen una alta expresión de moléculas co-estimuladoras CD80 y CD86. Funcionalmente participan en la eliminación de patógenos durante diversas infecciones a través de la activación del sistema de oxidación fosfato dinucleotido adenin-nicotinamida (NADPH) y en consecuencia la producción de la enzima iNOS y activación de especies reactivas de oxigeno (ROS). De esa forma los M1 tienen una robusta actividad anti-microbiana y anti-tumoral mediada por iNOS y ROS induciendo daño (Figura 3), retraso en la regeneración y cicatrización de tejidos. Para proteger a los tejidos de dicho daño la respuesta inflamatoria crónica es inhibida por mecanismos regulatorios que conllevan a la activación de macrófagos anti-inflamatorios M2 [62, 63]

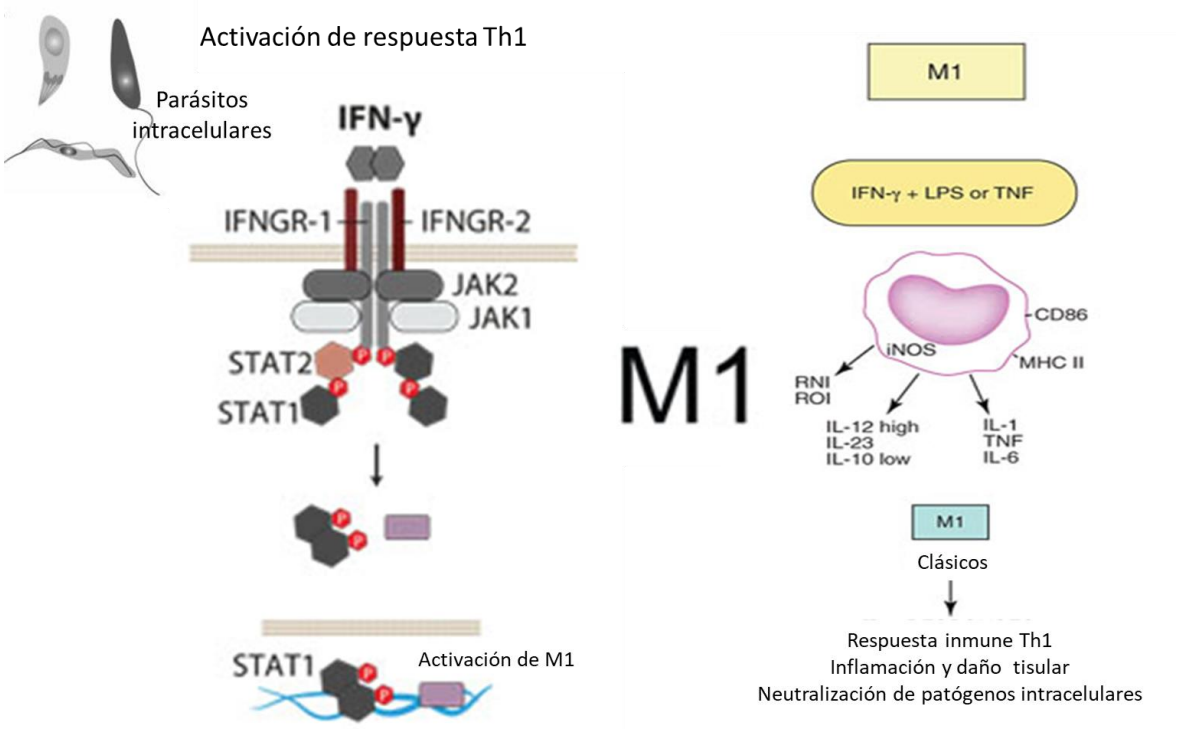

Figura 3. Al entrar el organismo en contacto con agentes patógenos intracelulares se inicia la activación de una respuesta inmune inflamatoria de tipo Th1 induciendo la producción de IFN-γ, el cual se une a receptores de IFN (IFNGR-1 y 2) en la superficie de las células para la activación de la vía de señalización de STAT1, estos receptores que son trans-membranales entran en contacto con las proteínas JAKs 1 y 2 que se encuentran de manera constitutiva en el citoplasma de las células, forman dímeros que son fosforilados y de esa forma se internalizan al núcleo de las células para la activación y transcripción de genes de M1 y amplificación de la respuesta Th1. Una vez activados los M1 por la vía de señalización STAT1, tienen la capacidad de producir citocinas como IL-12, 23, 6, 1 y TNF-α para la amplificación de la respuesta inmune Th1, y expresar marcadores de superficie como CD86 y MHCII, así como la producción de iNOS enzima que induce la producción de especies reactivas de oxígenos y óxido nítrico, para la eliminación de parásitos intracelulares, procesos que generan daño e inflamación tisular. Imagen modificada de Martínez FO y Gordon S [65].

1.6.2. Macrófagos M2

Los macrófagos anti-inflamatorios M2 son polarizados por citocinas tipo Th2 IL-4 e IL-13 vía STAT-6 por IL-4Rα). Así mismo los M2 tienen un perfil anti-inflamatorio, el cual se caracteriza por la producción de citocinas como IL-10 y TGF-β, también se caracterizan por la expresión de la enzima Arginasa-1, proteínas de la familia de las quitinasas YM1, YM2 y AMCase, moléculas tipo resistina como FIZZ1/Retnla/Relmα, FIZZ2/Retnlb/Relmβ, FIZZ3/Retn/Resistina y FIZZ4/Retnlg/Relmγ [66] y MMR/CD-206. Todos estos factores actúan en conjunto para promover la inmunidad en contra de parásitos helmintos y modular la inflamación (Figura 4). Funcionalmente tienen una potente capacidad fagocítica para la remoción de debridados y células apoptóticas, promoviendo la reparación y cicatrización tisular, a través de angiogénesis y fibrosis [67, 68].

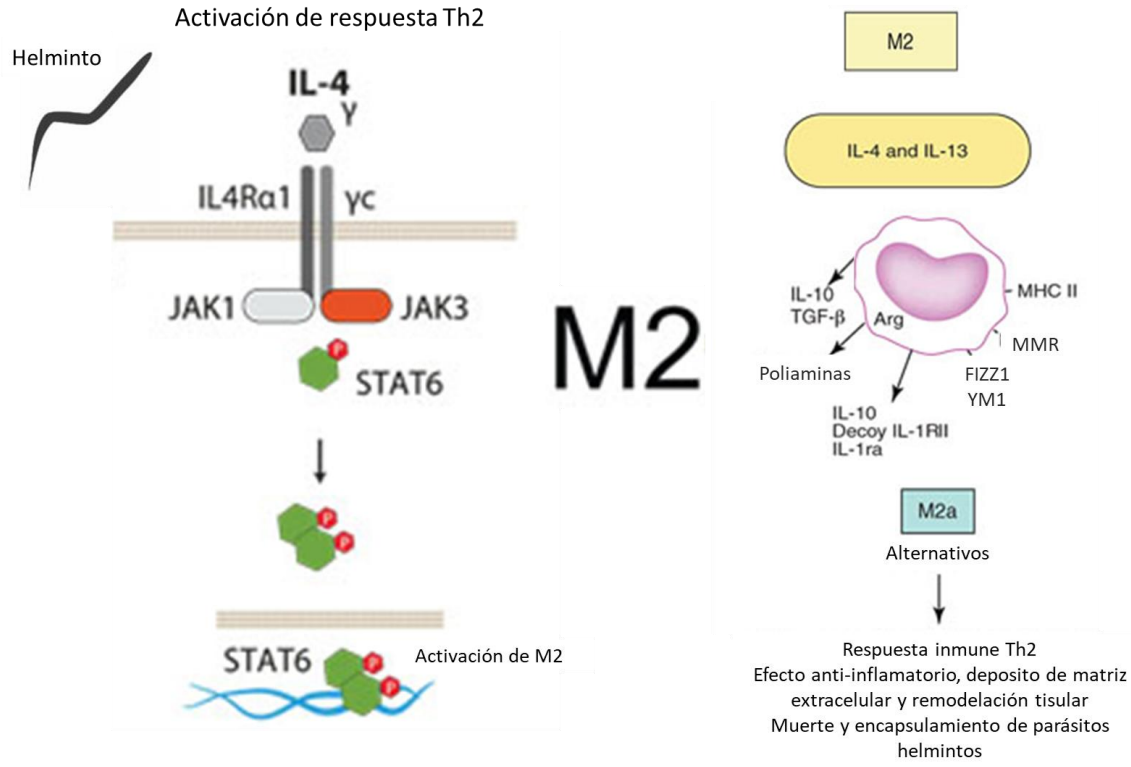

Figura 4. Al entrar el organismo en contacto con parásitos helmintos se inicia la activación de una respuesta inmune anti-inflamatoria de tipo Th induciendo la producción de IL-4 e IL-13, las cuales se une a receptores de la cadena α de IL-4 y γc en la superficie de las células para la activación de la vía de señalización STAT6, estos receptores que son trans-membranales entran en contacto con las proteínas JAKs 1 y 3 que se encuentran de manera constitutiva en el citoplasma de las células, forman dímeros que son fosforilados y de esa forma se internalizan al núcleo de las células para la activación y transcripción de genes de M2 y amplificación de la respuesta Th2. Una vez activados los M2 por la vía de señalización STAT6, tienen la capacidad de producir citocinas como IL-10, TGF-β e IL-1 para la amplificación de la respuesta inmune

Th2, y expresar marcadores de superficie como FIZZ1, YM1, MMR y MHCII, así como la producción de Arginasa-1 enzima que ayuda en la activación de los procesos de regeneración tisular como depósito de colágeno, elastina, producción de poliaminas y; junto con FIZZ1; en la producción de moco para la eliminación de helmintos. Imagen modificada de Martínez FO y Gordon S [65].

1.6.3. Activación de macrófagos clásicos y alternativos vía STAT1 y STAT6

Las proteínas STATs, originalmente descubiertas como proteínas de unión a DNA, son factores de trascripción latentes en el citoplasma de las células, activados por ligandos de señales extracelulares como citocinas, factores de crecimiento y hormonas. Las STATs son activadas mediante su fosforilación en el citoplasma de las células por las janus cinasas (JAKs) una familia de tirosin cinasas (TKs). Estas vías de señalización tienen funciones biológicas diversas que incluyen un papel en la diferenciación celular, proliferación, desarrollo, apoptosis e inflamación [69].

Debido a que existen cuatro tipos diferentes de moléculas JAK (JAK 1, 2, 3 y TYK 2) y siete miembros de la familia de las STATs (STAT1, 2, 3, 4, 5a, 5b y 6), combinaciones específicas de un receptor de citocina y la activación de las JAK/STATs puede llevar a una respuesta celular específica [70].

Las STATs en el citoplasma de las células se encuentran en forma de monómeros, una vez fosforiladas por las JAKs forman dímeros para ser traslocadas al núcleo y unirse al DNA de la célula e iniciar la transducción de genes específicos dependiendo del tipo celular [71].

Así linfocitos cooperadores tipo (Th)-1 son diferenciados a través de la fosforilación de STAT-1 por JAK1 y JAK2 o JAK1 y TYK3 moléculas activadas por la unión de IFN-γ a su receptor presente en la membrana celular, citocina que además también es producida por los mismos linfocitos Th1 una vez activados [70, 72].

Mientras que la fosforilación de STAT-6 por la activación de JAK1 y JAK3 a través de la unión de IL-4 e IL-13 a su receptor γC común en la membrana de las células, induce la diferenciación de linfocitos Th-2, que a su vez también son capaces de producir dichas citocinas una vez activadas [70, 72].

De tal forma que la señalización por STAT1 está estrechamente relacionada no sólo con la activación de la respuesta inmune Th-1 sino que también con la activación de M1 mediante la producción de IFN-γ, y en los cuales STAT1 también se encuentra de forma latente en el citoplasma para su diferenciación y transcripción de genes. Y de Igual forma la señalización de STAT6 se lleva a cabo en células M2 para la transcripción de genes asociados con esta población celular.[73-75].

1.7. Papel de los macrófagos durante diversas infecciones por helmintos

1.7.1. Nippostrongylus brasiliensis.

Este nematodo como muchos otros tiene una fase tisular, la cual migra de forma transitoria a través de pulmón ocasionando hemorragia e inflamación. El papel de la respuesta inmune en el control del daño pulmonar agudo se ha estudiado a través de un modelo de infección experimental con *N. brasiliensis* en ratones BALB/c, en el cual se encontró que la IL-17 contribuye en el proceso de inflamación, mientras que la señalización a través del receptor de IL-4 (IL-4R) reduce los niveles de IL-17, incrementando la expresión del factor de crecimiento tipo insulina 1 (IGF-1) e IL-10, con la consiguiente activación de M2, lo cual contribuyó a la rápida resolución del tejido dañado. Dicho estudio apunta hacía un papel esencial de la respuesta inmune Th2 y de M2 en la mediación del daño pulmonar [76]. En otro estudio usando los biomarcadores de FIZZ/Retn de los macrófagos alternativos, se observó que ratones Retnla$^{-/-}$ infectados con *N. brasiliensis* y *S. mansoni* presentaban una patología pulmonar y hepática intensificada, que indujo un proceso de fibrogénesis descontrolado derivado de la migración de los parásitos, así como disminución de la carga parasitaria a nivel intestinal e incremento de citocinas IL-4 e IL-13 de la respuesta inmune Th2, concluyendo que contrario a lo que se esperaba, el gen de Retnla inducido por la respuesta inmune Th2 suprime resistencia a la infección por nematodos gastrointestinales y fibrosis por regulación negativa dependiente de la respuesta inmune Th2, en donde la fibrosis puede estar asociada a un proceso de reparación descontrolado por la ausencia de FIZZ-1 [77]. En cuanto al papel de Ym1 en la infección por *N. brasiliensis*, biomarcador también de los M2, se observó que Ym1 inducía neutrofilia y daño pulmonar agudo caracterizados por destrucción alveolar y hemorragia asociados a la presencia de neutrófilos, ya que al inyectar anti-Ym1 a ratones BALB/c la población de neutrófilos tanto en lavados pulmonares como en tejido pulmonar disminuía de manera considerable al día 2 y 4 posteriores a la infección, así como la patología pulmonar, lo cual se asoció a un incremento en la población de macrófagos al día 4 consistente con la disminución del daño pulmonar, dichos datos sugieren que aún en ausencia de Ym1 los M2 siguen ejerciendo su papel reparador que podrían estar llevando a cabo a través de sus diversos mecanismos independientes de Ym1 [78], otros estudios también han confirmado la importancia de los neutrófilos como células implicadas en la mediación de la reparación de tejido además de la neutralización de parásitos, ya que su presencia se ha asociado a la activación de M2 [79]. También se ha estudiado la importancia de la IL-9 en la activación de las células linfoides de la inmunidad innata de tipo 2 (ILC2s) y de estas a su vez en la reparación de tejido pulmonar durante la infección por *N. brasiliensis*, ya que ratones IL-9R deficientes mostraron disminución en la población celular de ILC2s y de anfiregulina; un

miembro de la familia del factor crecimiento epidermal el cual promueve regeneración de epitelio bronquio alveolar; dando como resultado decremento en producción de IL-5 e IL-13 y como consecuencia deficiencia en la restauración del tejido dañado, todo ello desencadenado por la ausencia de IL-9. Esta incapacidad de regeneración tisular se asoció a una producción disminuida de los factores involucrados en la reparación de macrófagos Arg-1, Retnla y Ym1/Chi3l3. De esta forma las células de la inmunidad innata juegan un papel importante para la posterior reparación tisular por parte de los macrófagos, que de nuevo demuestran ser importantes para que dicho proceso se lleve a cabo de forma adecuada [80]. Sin embargo también las ILC2s están implicadas en la reparación de tejido de forma directa ya que se ha visto que expresan Arginasa-1 de manera constitutiva con capacidad de inducir regeneración tisular durante procesos de inflación pulmonar en ausencia de M2 [81].

1.7.2. Schistosoma mansoni.

Durante la infección por este nematodo se demostró que AAM son esenciales durante la esquistosomiasis para la protección contra el daño tisular a través de la disminución en la inflamación desencadenada por la presencia de huevos de *S. mansoni* en tejido hepático. Para probar este efecto se usaron ratones $LysM^{cre}IL\text{-}4^{-/flox}$ e IL-4 deficientes, los cuales mostraron una incapacidad en la activación de AAM, con polarización de macrófagos hacía un perfil clásico productores de NOS2, que indujeron formación de granulomas hepáticos más grandes en comparación con ratones WT, los cuales presentaron una activación alternativa de macrófagos predominante, con producción de Arginasa-1 y formación granulomas hepáticos más pequeños [65] y resultados muy similares fueron encontrados por Kevin M. y col que usando la cepa de ratones $IL\text{-}4Ra^{flox/}LysM^{Cre}$, demostraron que AAM son requeridos para suprimir la respuesta patogénica Th1/CAM, pero sin tener un impacto significativo en el desarrollo de la fibrosis a pesar de que aparentemente la fibrosis si fue mayor en ratones deficientes [82]. Aunque estos datos son contrarios a unos publicados anteriormente, en los cuales a nivel pulmonar la inducción de IL-4 e IL-13 produce formación de granulomas que se encuentran rodeando los huevos de *S. mansoni*; que comparados con los granulomas formados en ausencia de IL-4 e IL-13; resultan ser más grandes. Estos datos sugieren que un estímulo exacerbado de la respuesta inmune Th2 puede estar involucrada en el desencadenamiento de daño tisular más que en la reparación, pero de manera interesante este daño se encuentra asociado a un incremento en el reclutamiento de eosinófilos, implicándolos como las principales células en generar el daño pulmonar ocasionado por la

formación de granulomas, sin hacer correlación alguna con los M2, a pesar de que este tipo celular es también un componente importante durante la activación de la respuesta inmune Th2 [83]. En este aspecto se llevó a cabo otro estudio en tejido pulmonar en el que los resultados confirman una vez más que los M2 siguen teniendo una función benéfica. Usando ratones FHL2-knockout; siendoFHL2 una proteína inducida en macrófagos bajo el estímulo de citocinas como IL-4 e IL-10; los macrófagos derivados de médula ósea (BMMs) mostraron un fenotipo alternativo reducido, aún bajo el estímulo de IL-4, así como incremento en el número de granulomas pulmonares, con decremento en la expresión de marcadores alternativos y en consecuencia una respuesta Th1 exacerbada, concluyendo que FHL2 es importante para a activación de M 2y en consecuencia para la reparación del daño ocasionado por la presencia de *S. mansoni* en tejido pulmonar, del mismo modo se ha visto que la activación de M2 dependiente de IL-13, es importante para la resolución de fibrosis e inflamación pulmonar [84, 85].

A nivel intestinal se ha visto que la Arginasa-1 producida por M2 juega un papel crucial en los procesos inflamatorios inducidos por la presencia de los huevos de *S. mansoni*. En este estudio la ausencia de IL-4Rα ocasiono decremento en la producción de Arginasa-1 a nivel intestinal, la cual parece ser importante para reducir el daño intestinal ya que al administrar BEC a ratones WT; un inhibidor competitivo que específicamente bloquea las funciones de Arg-1 y 2; presentaron incapacidad para eliminar los huevos de *S. mansoni* y por consiguiente la patología intestinal incrementó, lo cual se vio reflejado en un incremento de mortalidad y morbilidad en comparación con ratones no tratados. Para poder medir de forma directa la importancia de Arg-1 en la patología intestinal se infectaron ratones Arg-$1^{-/-}$, los cuales desarrollaron diversas lesiones hemorrágicas dentro de la mucosa intestinal, en comparación con ratones WT que no las desarrollaron. Los datos de este estudio en conjunto sugieren que la producción de Arg-1 por parte de los macrófagos es importante para la neutralización de los huevos de *S. mansoni* y en consecuencia para disminuir la patología intestinal por la ausencia del parásito, ya que la ausencia de Arg-1 también indujo polarización de la respuesta inmune hacía un perfil inflamatorio desencadenando mayor patología [86]. También al inhibir la respuesta Th1 durante la infección con *S. mansoni*, se ha visto que la población de AAM y sus mecanismos de reparación resultan más eficientes, ya que al usar ratones CD14 deficientes; correceptor de TLR4 que desencadena la respuesta Th1 y activación de CAM; la población de AAM incremento de manera significativa, así como de células CD4+IL-4, IL-5, e IL-13+ y CD4+Foxp3+IL-10+, en los cuales la formación de granulomas hepáticos resultaron ser más

pequeños; asociado con un incremento en el depósito de colágeno; comparados con los granulomas de ratones WT infectados. Tales efectos se relacionaron con un incremento en la señalización de STAT-6, sugiriendo que la ausencia de CD-14 tiene un efecto en la vía IL-4Rα/STAT-6 para la polarización de macrófagos durante la infección por este nematodo. [87, 88]. Otros estudios han confirmado este papel reparador de los macrófagos, que resulta ser poco benéfico para el hospedero al inhibir alguna citocina o factor característico de los M2 y en los cuales los resultados obtenidos demuestran que la presencia de esta población celular y sus factores son indispensables para la adecuada resolución de lesiones ocasionadas por la formación del granuloma de los huevos de *S. mansoni* en los diferentes órganos a través de los cuales migra [89, 90].

1.7.3. Heligmosomoides polygyrus.

Diversas poblaciones celulares, citocinas, vías de señalización, factores quimiotácticos, etc. se han asociado con la activación de M2 para que puedan cumplir con sus funciones de reparación, sin embargo un estudio reciente además vinculó la importancia de la producción de anticuerpos en la activación de M2. Dicho estudio se llevó a cabo durante la infección con *Heligmosomoides polygyrus bakeri* (*Hp*), en el cual se observó que anticuerpos específicos para *Hp* dependientes del complemento y FcRγ inducían adherencia de macrófagos a las larvas de *Hp in vitro*, dando como resultado completa inmovilización de los parásitos. También se observó que los anticuerpos tenían la capacidad de reprogramar a los macrófagos para la expresión de genes asociados a los procesos de reparación. Ambos mecanismos pudieron llevarse a cabo de manera independiente de la señalización de IL-4Rα, dando como resultado un nuevo mecanismo de activación alternativa de los macrófagos dependiente de anticuerpos y como mediadores del efecto antihelmíntico y de reparación de los macrófagos [91]. Como la gran mayoría de los helmintos *H. polygyrus* tiene la capacidad de migrar a través de diversos órganos y un estudio en particular se enfocó en identificar la densidad, estatus de la polarización y distribución de los macrófagos en tejido cardiaco, ya que si bien es sabido que los macrófagos residentes en los diversos tejidos tienen un papel homeostático muy importante; en tejido cardiaco no es del todo claro. Para poder dilucidar esta cuestión se hizo un reto inmunológico con *H. polygyrus* en ratones, los cuales mostraron una polarización de la población de macrófagos hacia un fenotipo alternativo caracterizado por la expresión de receptores como Ym-1, RELM-α y CD206 (receptor de manosa), seguido de un incremento en el depósito de colágena, que bajo condiciones normales, es decir; sin infección; no había un predominio en la población de macrófagos, ya que expresaban marcadores tanto de un perfil clásico (IL-1β, TNF,

CCR2) como alternativo (Ym1, Arg1, RELMα e IL-10) y con función fagocítica; pero no fue sino hasta que se hizo el reto inmunológico que se diferenciaron hacia el fenotipo alternativo, que condujo hacía fibrosis del tejido cardiaco. Estos datos indican que la polarización de los macrófagos residentes de tejido cardiaco, depende del estímulo inmunológico que se les dé y que no hay una predominancia en condiciones basales. Por otro lado aún falta estudiar más a fondo el papel reparador de AAM en tejido cardíaco, debido a que el efecto observado después de su activación fue la fibrosis, mecanismo a través del cual M2 ejercen su función reparadora, pero no se determinó si este efecto era positivo o negativo para el hospedero [92].

1.7.4. Trichinella spiralis.

Los macrófagos tienen una alta plasticidad y heterogeneidad, sus funciones se llevan a cabo con base a los requerimientos del microambiente y los procesos de obesidad son conocidos como un tipo de inflamación del tejido adiposo que en muchos casos conlleva a resistencia a la insulina y descontrol de la glucosa, en este aspecto los M2 tienen una función muy importante mediando dichos procesos inflamatorios. Durante la infección con *T. spiralis* M2 son activados en respuesta a la infección y su presencia tiene un efecto benéfico al disminuir la tolerancia a la glucosa en ratones obesos, los cuales muestran un decremento de la glucosa en sangre periférica, seguido de un incremento en la expresión de marcadores de activación alternativa como Arg1, CD206 e IL-10 y muerte de adipocitos; de tal forma que la presencia de los M2 activados por este parásito en procesos de obesidad tiene un efecto benéfico; comprobando una vez más la eficaz funcionalidad de los M2 en la mediación de los procesos inflamatorios y de reparación, pero ahora en tejido adiposo [93]. Durante otros procesos inflamatorios como la colitis, se ha demostrado que las proteínas de excreción-secreción (SE) de los parásitos juegan un papel importante en los procesos de inmunomodulación, en donde rTsP53 (proteína recombinante de 53k Da de *T. spiralis*) un componente de las proteínas de ES de *T. spiralis,* tiene efectos inmunológicos en la polarización de la respuesta inmune hacía Th2 durante la colitis experimental en ratones. La inducción de la colitis experimental, seguida del tratamiento con rTsP53; además de la polarización de la respuesta inmune, conlleva a un decremento de la inflamación del colón y expresión de marcadores de activación alternativa Arg1, FIZZ1, TGF-β e IL-10, que en conjunto disminuyen los procesos patológicos derivados de la inflamación del colón, teniendo la activación de los M2 y la expresión de sus factores una implicación muy importante en dichos efectos anti-inflamatorios [94].

1.7.5. Taenia crassiceps.

Otro nematodo en el cual se han estudiado ampliamente sus efectos durante la colitis experimental y el cáncer asociado a colitis, es el cisticerco de *T. crassiceps*, cuya infección se ha visto que al igual que rTsP53 de *T. spiralis*, tiene efectos inmunomoduladores que ayudan a disminuir los efectos inflamatorios y tumorigénicos asociados a la colitis y al cáncer asociado a colitis; en donde los M2 tienen un papel central en la mediación de dichos procesos. Los efectos inmunomodulares de la infección con el cestodo de *T. crassiceps* han demostrado tener una implicación importante en la disminución de ambas patologías en donde M2 tiene un papel activo induciendo la expresión de sus factores reparadores Arg1, Ym1 y Fizz1 y por consiguiente el depósito de colágena en intestino; pero sin producir fibrosis ni incremento en la patología intestinal. También se observó una reducción importante del infiltrado inflamatorio del colón y de la hemorragia ocasionada por la colitis durante la infección con *T. crassiceps* [95, 96]. De forma muy similar se han observado estos efectos durante la inducción de la encefalitis autoinmune experimental (EAE), patología inflamatoria de sistema nervioso central, que en presencia del cisticerco de *T. crassiceps* disminuye de manera considerable y sólo 50% de los ratones infectados mostraron síntomas de EAE, que fueron significativamente menos severos comparados con ratones no infectados. La ausencia o disminución de la sintomatología en los ratones infectados se asoció principalmente a la producción de citocinas anti-inflamatorias, así como a una alta expresión de marcadores de activación alternativa [97].

1.7.6. Trichuris muris.

Poco se ha estudiado en cuanto al papel de M2 durante la infección por el nematodo intestinal de *Trichuris muris*, pero se sabe que también tiene la capacidad de inducir una respuesta inmune Th2 y por consiguiente la activación de esta población de macrófagos; aunque a diferencia de otras helmintiasis, este tipo de respuesta inmune genera resistencia a la infección por, asociada también a la presencia de M2. En cuanto a los efectos reparadores de M2 se demostró que Arg1 no es esencial para que sean llevados a cabo, ya que su ausencia no produjo cambios importantes en la patología intestinal. Sin embargo, siempre es importante recordar que existen otros mecanismos a través de los cuales se llevan a cabo los procesos de reparación y que no fueron tomados en cuenta en este estudio, así como tampoco se profundizo en, si el efecto reparador de los macrófagos este siendo la condicionante para que la eliminación de los parásitos no se lleve a cabo de manera adecuada [98].

1.7.8. Toxocara canis.

Para determinar la respuesta inmune que se desencadena durante la infección por *T.* canis se han llevado a cabo diversos estudios, en los cuales se ha observado la activación de una respuesta inmune tipo Th2 caracterizada tanto por un incremento en la producción de citocinas IL-4, IL-5, IL-13 y anticuerpos IgG-1a, IgG4a e IgE, así como de eosinófilos en sangre periférica y formación de granulomas eosinofílicos a nivel pulmonar y hepático [5]. A pesar de que *T. canis* tiene la capacidad de migrar por diversos órganos y de que la respuesta inmune desencadenada es la encargada de activar a M2 es poco lo que se ha estudiado de este nematodo en cuanto a reparación del tejido por parte de esta población celular en comparación con *N. brasiliensis* y sólo se ha llevado a cabo un estudio en tejido pulmonar. En dicho estudio usando ratones deficientes en la molécula de STAT6, se asoció la persistencia de inflamación y lesiones hemorrágicas pulmonares ocasionadas por la migración de las larvas de *T. canis*, a una débil respuesta inmune Th2 dependiente de la vía de señalización de STAT6, ya que ratones $STAT6^{+/+}$ BALB/c infectados con *T. canis* desarrollaron una fuerte respuesta inmune Th2 produciendo altos niveles de IgG1a, IgE e IL-4, y en consecuencia reclutamiento de M2 mostrando una patología pulmonar moderada. Mientras que ratones $STAT6^{-/-}$ BALB/c montaron una débil respuesta Th2, sin reclutamiento de M2 y en consecuencia el desarrollo de una patología pulmonar más severa; lo cual se asoció principalmente a la ausencia de dicha población de macrófagos, que en otras infecciones por helmintos se ha visto que tienen un efecto reparador [24].

A pesar de que los M2 podrían jugar un papel importante en la reparación de tejido durante la infección por *T. canis*, estudios en pacientes infectados han demostrado que se desencadenan reacciones inflamatorias y de hipersensibilidad crónicas en vías respiratorias, lo cual se ha asociado principalmente con la presencia de eosinófilos, encargados de rodear a las larvas formando granulomas alrededor de ellas con la finalidad de neutralizarlas, pero desencadenando efectos dañinos que resultan ser desfavorables para las personas [99-101], mientras que los M2 no tienen una implicación en dichos procesos tanto como los eosinófilos, ya que su papel no se ha estudiado ampliamente durante la infección por *T. canis* y en otras helmintiasis en general se ha centrado en la reparación de tejidos.

2. JUSTIFICACIÓN

Al ser *T. canis* un parásito que genera una importante zoonosis en humanos y el desarrollo de patologías pulmonares con un alto impacto en la salud de las personas infectadas es imprescindible conocer los mecanismos asociados con la patología pulmonar y la subsiguiente regeneración del tejido, asociados con la respuesta inmune desencadenada durante la infección, para lo cual proponemos a los macrófagos como una de las principales células encargadas de sobrellevar dichos procesos. Por un lado se espera que los macrófagos M1, sean las principales células involucradas en procesos patológicos desencadenados a nivel pulmonar, mientras que los M2 serán los encargados de contrarrestar sus efectos, de tal forma que un balance entre ambas poblaciones celulares promoverá la óptima regeneración tisular del tejido pulmonar.

3. HIPÓTESIS

La ausencia de STAT1 estará asociada a una pronta involución de lesiones pulmonares, derivada de la activación oportuna de macrófagos M2, mientras que en ausencia de STAT6 estará asociada a la persistencia de lesiones tisulares y reparación tardía del tejido pulmonar por la presencia de M1 en la infección por *T. canis*.

4. OBJETIVOS

4.1. Objetivo general

Determinar si la reparación de tejido pulmonar se encuentra asociada a la producción de factores de activación alternativa de los macrófagos cuya activación es dependiente de STAT-6, y sí la persistencia de lesiones hemorrágicas se asocia a la producción de factores de activación clásica de los macrófagos, cuya activación es dependiente de STAT-1.

4.2. Objetivos particulares

- Cuantificar el número de parásitos en los diferentes órganos por los cuales migra el parásito, con la finalidad de establecer el modelo de infección en ratones WT, STAT1$^{-/-}$ y STAT-6$^{-/-}$
- Evaluar a través del estudio macroscópico de tejido pulmonar las diferencias en la presentación de lesiones hemorrágicas derivadas de la migración del parásito por este órgano en ratones STAT1$^{-/-}$ y STAT-6$^{-/-}$ en comparación con ratones WT.
- Confirmar si el mismo patrón de las lesiones hemorrágicas es también observado a nivel histológico en las diferentes cepas de ratones.
- Determinar si el infiltrado inflamatorio en tejido pulmonar, además de la migración del parásito, puede estar asociado con la persistencia de lesiones hemorrágicas en ratones STAT-6$^{-/-}$, o con la reparación temprana del tejido en ratones STAT-1$^{-/-}$ comparados con ratones WT.
- Determinar si el proceso temprano de reparación del tejido pulmonar en ratones STAT-1$^{-/-}$ podría estar asociado con el desencadenamiento de una respuesta inmune antiinflamatoria relacionada con la activación de AAM y en ratones STAT-6$^{-/-}$ a la respuesta inmune pro-inflamatoria con la activación de CAM.
- Medir la expresión de factores de activación alternativa y/o clásica de los macrófagos en las diferentes cepas de ratones relacionados con la reparación o persistencia de lesiones pulmonares.
- Confirmar si los macrófagos AMM son las células involucradas en el proceso de reparación y los CAM las células asociadas al daño de tejido pulmonar a través de su eliminación.

5. MATERIALES Y MÉTODOS

5.1. Animales

Ratones machos de 8 a 10 semanas de edad de las cepas WT, STAT1$^{-/-}$ y STAT6$^{-/-}$ con fondo genético BALB/c, fueron obtenidos del centro de recursos de laboratorio animal Jackson y mantenidos en un ambiente libre de patógenos, en las inmediaciones del bioterio de la FES-Iztacala UNAM, de acuerdo con los lineamientos establecidos en la NOM-062-ZOO-1999, 2002.

5.2. Obtención de huevecillos e infección

Gusanos adultos de *T. canis* fueron obtenidos de intestinos de cachorros infectados de manera natural. Las hembras de los gusanos fueron diseccionadas para aislar huevecillos inmaduros del útero y mantenidos en agua destilada. Posteriormente se realizaron 2 lavados de diez minutos en una solución de NaHCl al 1% y se centrifugaron a 1500rpm. Se retiró el sobrenadante y el sedimento fue lavado 2 veces más con agua destilada y los huevecillos fueron mantenidos en cajas Petri a 28°C durante un mes, con agitación diaria hasta que se desarrolló el segundo estadio larvario de *T. canis*, dicho proceso de maduración fue monitoreado bajo la luz del microscopio y una vez confirmado, los ratones antes mencionados fueron infectados con 500 huevos larvados, administrados de forma intragástrica mediante una sonda para neonatos tipo Foley

5.3. Cinética de migración del parásito

Los animales fueron sacrificados a 0 (no infectados), 2, 4, 7, 14, 21, 28 y 60 días post infección (dpi). Pulmones, hígado, músculo y cerebro se cortaron en trozos pequeños y se colocaron en tubos que contenían ácido clorhídrico al 1% (J.T. Baker; Edo. De Mex, México) y pepsina al 3% (Sigma Aldrich; St Louise; EE. UU.). Para facilitar la liberación de parásitos L2 de los diferentes órganos, los tejidos se incubaron durante 48 horas a temperatura ambiente en agitación. Después de la incubación, las muestras se centrifugaron a 1500 rpm durante 10 minutos, el sedimento se recuperó, se reconstituyó y se fijó con formalina al 10% (Sigma Aldrich; Ciudad de México, México), y el número de L2 se cuantifico bajo el objetivo 10x de un microscopio óptico (Motic B5 Serie Profesional).

5.4. Estudio microscópico.

Se tomaron pulmones completos de los ratones, se lavaron con solución salina, se fotografiaron con un objetivo 4x usando un microscopio estereoscópico (ZEIGEN), y se contaron las lesiones hemorrágicas en la superficie de los pulmones.

5.5. Histología y tinción de Hematoxilina-Eosina (H&E)

Los pulmones se perfundieron a través de la tráquea y se fijaron con alcohol absoluto, seguido de su inclusión en parafina, se hicieron cortes de 4 µm de espesor y se tiñeron con H&E para su análisis histológico. Las características morfológicas se evaluaron utilizando un microscopio óptico (LAS V4.9, Leica), y se realizó un análisis morfométrico con el programa Leica para determinar la superficie total y calcular el área de infiltrado inflamatorio por µm2.

5.6. Inmunohistoquímica (IHQ) e Inmunofluorescencia (IMF)

Se desparafinaron secciones de pulmón de cuatro micras de grosor y se incubaron con 10x DIVA Decloaker (Biocare Medical; CA, EE. UU.) para la recuperación antigénica a una dilución 1:10. Para IHQ, los portaobjetos se lavaron con PBS (3x 5 min) y la inhibición de la peroxidasa se realizó con peróxido de hidrógeno al 10% durante 30 min, los portaobjetos se lavaron con PBS (3x 5 min) y se bloquearon con PBS BSA al 3% de durante 1 hora a temperatura ambiente. Los portaobjetos se incubaron con el anticuerpo primario purificado anti-elastina hecho en conejo a una dilución 1: 200 (Abcam; MA, EE. UU.) o anti-MMP9 hecho en cabra a una dilución 1: 100 (Santa Cruz; CA, EE. UU.) a 4 ° C durante la noche. A continuación, los portaobjetos se lavaron con PBS (3x 5 min) y el anticuerpo secundario anti-conejo hecho en ratón a una dilución 1: 1,500 (Biolegend; CA, EE. UU.) o anti-cabra hecho en ratón a una dilución 1: 1,500 (Biolegend; CA, EE. UU.) fue añadido, dejándose incubar durante 1 hora a temperatura ambiente. Los portaobjetos se lavaron e incubaron durante 5 minutos con 50 µl de kit de cromógeno de diaminobencidina (DAB) (Biocare Medical; CA, EE. UU.) de acuerdo con las instrucciones del fabricante. Finalmente se realizó una contratinción con hematoxilina de Harris. Las fotografías se obtuvieron con un microscopio óptico (Axio Vert. A1, Carl Zeiss) con el objetivo 40x y se analizaron con el programa ImageJ. Para IF, los portaobjetos se lavaron con PBS (3x 5 min) y la permeabilización de membranas se realizó con PBS Tritón al 2% (Reasol; Ciudad de México, México). Las secciones se lavaron con PBS (3x 5 min) y se bloquearon con PBS BSA al 3% durante 1 hora a temperatura ambiente. Se incubaron diferentes secciones de tejido con anticuerpos cargados con el fluorocromo FITC hecho en conejo anti-iNOS a una dilución 1: 100 (Cell Signaling; MA, EE. UU.) o conejo anti-Ym1 a una dilución 1: 100 (Stem Cell, CA; Canadá) toda la noche a 4 ° C. A continuación, los portaobjetos se lavaron con agua destilada y se montaron con una gota de medio de montaje Fluoroshield con DAPI (Abcam; MA, EE. UU.) por sección de tejido. Las IF se analizaron utilizando un microscopio Axio Vert A1 (Zeiss).

5.7. ELISA

Se colectó sangre periférica de la vena lateral de la cola de los ratones, se centrifugó a 2500 rpm durante 10 minutos y se obtuvo el suero en el cual se analizó la producción de anticuerpos específicos IgG1 e IgG2a anti-*T. canis* en placas previamente sensibilizadas (1 µg/ml) con antígenos excretados-secretados de *T. canis*, obtenidos de acuerdo a los protocolos ya descritos [102]. Después de la incubación durante la noche a 4 ° C, las placas se lavaron con PBS Tween-20 al 0,05% (Sigma; St. Louise, EE. UU.) y se bloquearon con PBS BSA al 1% (Biowest). Se hicieron diluciones en serie 1:100 de las muestras de suero. Los anticuerpos unidos se detectaron después de la incubación con IgG1 o IgG2a anti-ratón de rata conjugados con HRP (Zymed; San Francisco, EE. UU.) y se leyeron en un lector de microplacas a 405 nm (Multiskan Ascent, Thermo Labsystems). Los resultados se expresan como la dilución máxima de suero en la que se detectó la densidad óptica (DO). Los niveles séricos de IFN-γ, TNF-α, IL-4, IL-13 e IL-10 se midieron por ELISA (Peprotech; Ciudad de México, México) de acuerdo con las instrucciones del fabricante.

5.8. RT-PCR

Los pulmones se colocaron en tubos Eppendorf de 1,5 ml que contenían TRIzol (Invitrogen; CA, EE. UU.) y perlas de óxido de circonio de 0,5 mm de diámetro (Next Advance) para su procesamiento en un digestor de tejiddos (Next Advance). El ARN se extrajo mediante la técnica del cloroformo; una vez cuantificado, 1 µg del producto se transcribió inversamente usando el kit Superscript II First Strand Synthesis (Invitrogen; CA, EE. UU.). Los genes utilizados para su amplificación y las temperaturas de fusión se describen en la Tabla 1. Los productos obtenidos se mezclaron con buffer de carga que contenía SYBR Green para su amplificación y observación en un gel de agarosa al 1,5% (certificado por IBI Scientific en biología molecular) con un sistema de documentación de gel DocTM-EZ. Las imágenes se analizaron con ImageJ y los valores de expresión fueron normalizados con dehidrogenasa gliceraldehído 3-fosfato (GAPDH), como control.

Tabla 1. Genes utilizados y sus respectivas secuencias para determinar respuesta inmune y activación de macrófagos

Gen	Temperatura (°C)	Ciclos	Secuencia
Control			
GAPDH	54	35	F- CTC ATG ACC ACA GTC CAT GC R-CAC ATT GGG GGT AGG AAC AC
Citocinas Th1			

IFNγ	57	35	F-AGC GGC TGA CTG AAC TCA GAT TGT AG R-GTC ACA GTT TTC AGC TGT ATA GGG
TNFα	59	35	F-GGC AGG TCT ACT TTG GAG TCA TTGC R-ACA TTC GAG GCT CCA GTG AAT TCG
Citocinas Th2			
IL-4	58	35	F-CGAAGA ACA CCA CAG AGA GTG AGCT R-GAC TCA TTC ATG GTG CAG CCT ATCG
IL-10	56	35	F-ACC TGG TAG AAG TGA TGC CCC AGG CA R-CTA TGC AGT TGA AGA TGT CAA A
Marcadores M2			
Arg1	54	35	F-CAG AAG AAT GGA AGA GTC AG R-CAG ATA TGC AGG GAG TCA CC
FIZZ1	62	35	F-GGTCCCAGTGCATATGGATGAGACCATAGA R-CACCTCTTCACTCGAGGGACAGTTGGCAGC
Ym1	56	35	F-TCACAGGTCTGGCAATTCTTCTG R-TTTGTCCTTAGGAGGGCTTCCTC
TGF-β	60	35	F-GCCCTTCCTGCTCCTCAT R-TTGGCATGGTAGCCCTTG
Marcadores M1			
iNOS	65	35	F-CTGGAG GAG CTC CTG CCT CATG R-GCA GCA TCC CCT CTG ATG GTG

F, forward; R, reverse primer.

5.9. Citometría de flujo

Los pulmones obtenidos a 7 dpi se perfundieron con solución salina para la obtención de células. Se lisaron los glóbulos rojos y se contaron las células vivas por exclusión con azul de tripano (Countess II FL ThermoFisher). Las células se tiñeron para la expresión de marcadores de superficie usando los siguientes anticuerpos: anti-F4/80-Pacific blue o APC, anti-CD86-PE/Cy7, anti-CD206 (MMR)-PerCP o FITC y anti-IL4Rα-PE (todos de Biolegend; CA, EE. UU.). Las muestras se incubaron durante 30

minutos a 4°C en buffer de FACS (BD®). Las muestras se adquirieron con FACSAria Fusion (BD®) o Attune NxT (ThermoFisher) y se analizaron con el software FlowJo (versión 10.0.7).

5.10. Depleción de macrófagos

Depleción de macrófagos con liposomas de clodronato.

Administramos liposomas cargados de clodronato por vía intratraqueal de acuerdo con las instrucciones del fabricante (Formumax; CA, EE. UU.) para eliminar los macrófagos de tejido pulmonar. Dos días después del tratamiento con liposomas, los ratones se infectaron como se describió antes. Al día cuatro después del primer tratamiento, se administró una segunda dosis de 100 µl de liposomas cargados con clodronato o con PBS al grupo correspondiente. Al día 4 pi, los animales fueron sacrificados.

5.11. Análisis estadístico

Las diferencias entre los grupos se determinaron mediante la prueba t de Student no pareada de dos vías o el análisis de varianza de dos vías (ANOVA), y una post prueba de Holm-Sidak múltiple, y se tomaron como las medias ± SEM. Todos los análisis estadísticos se determinaron utilizando GraphPad Prism v 6.0, con un valor de "P" o menos de 0.05 considerado significativo (* $P < 0.05$). Todos los experimentos se llevaron a cabo de manera independiente al menos dos veces.

6. RESULTADOS

6.1. Carga parasitaria y cinética de migración

Para evaluar la susceptibilidad (presencia) o resistencia (ausencia) a las larvas de *T. canis*, ratones WT, STAT1$^{-/-}$ y STAT6$^{-/-}$ fueron infectados vía oral con 500 huevecillos larvados y se determinó la cinética de la carga parasitaria en hígado, pulmón, cerebro y músculo. Los ratones WT mostraron la mayor carga de parásitos en hígado y pulmones al inicio de la infección (4 dpi; Figura 5a, b) la cual disminuyó posteriormente. En cerebro y músculo resultó ser menor a los 4 dpi pero con un incremento gradual durante los siguientes 56 dpi (Figura 5c, d). Aunque los ratones STAT1$^{-/-}$ y STAT6 $^{-/-}$ mostraron los mismos patrones de migración larval que los ratones WT, la carga parasitaria en ambas cepas fue menor en comparación con los ratones WT. Estos datos sugieren que la ausencia de STAT1 y STAT6 no afectó el patrón de migración de las larvas y que ambas moléculas de STAT son cruciales para eliminar las etapas tisulares de *T. canis*, lo que hace que los ratones STAT1$^{-/-}$ y STAT6$^{-/-}$ sean menos susceptibles a Infección por L2 de *T. canis*.

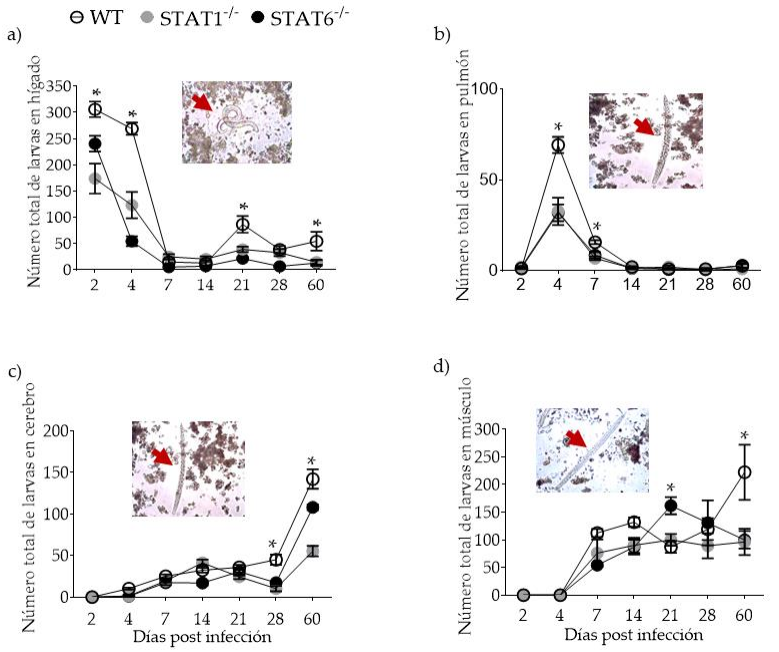

Figura 5. Cinética de la migración de parásitos. Después de la infección con 500 L2 nematodos de *T. canis*, los parásitos se contaron en hígado (a), pulmón (b), cerebro (c) y músculo (d), de ratones WT (círculos blancos sólidos), STAT1$^{-/-}$ (círculos grises sólidos) y STAT6$^{-/-}$ (círculos negros sólidos) a 2, 4, 7, 14, 21, 28 y 60 dpi. Las flechas rojas indican la presencia de larvas en cada tejido durante el recuento. Los datos se muestran a partir de dos experimentos independientes como la media ± SEM (n = 6 por grupo); ANOVA de dos vías con post prueba de Tukey; * P <0.05 comparando ratones WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$, a los mismos tiempos de infección.

6.2. Lesiones hemorrágicas e infiltrado inflamatorio en tejido pulmonar

Una vez confirmada la migración de L2 *T. canis* a través de los pulmones, se evaluó la patología pulmonar durante la infección aguda. Observamos el desarrollo de numerosas lesiones hemorrágicas en el parénquima pulmonar de ratones WT a 4 y 7 dpi, que disminuyeron

gradualmente con el tiempo (Figura 6a, b). Después de la infección con L2 *T. canis*, los ratones STAT1 $^{-/-}$ mostraron menos y más pequeñas lesiones en los pulmones a 4 y 7 dpi en comparación con los ratones WT; las lesiones pulmonares no fueron detectables a 28 dpi en los ratones STAT1$^{-/-}$ (Figura 6a, b). Sin embargo, los ratones STAT6$^{-/-}$, a pesar de tener menos larvas que los ratones WT (y similar a la carga parasitaria de los ratones STAT1$^{-/-}$), mostraron numerosas lesiones hemorrágicas en los pulmones que persistieron hasta 60 dpi (final del experimento) (Figura 5a, b). El examen histológico de los pulmones reveló inflamación peribronquial y perivascular durante la infección aguda (4-7 dpi) que disminuyó a medida que la infección progresaba en ratones WT y STAT1$^{-/-}$. La presencia de eritrocitos en el espacio alveolar y de larvas rodeadas de infiltrado inflamatorio (Figura 5c-e) se correlacionaron con las lesiones hemorrágicas observadas en el estudio macroscópico. Curiosamente, el análisis histológico de los pulmones de ratón STAT6$^{-/-}$ reveló un marcado infiltrado inflamatorio que persistió por más tiempo (4 a 60 dpi), lo que sugiere un proceso de regeneración retardada, a pesar de la eliminación del parásito. Estos hallazgos sugieren que la regeneración rápida o tardía de los pulmones en ratones STAT1$^{-/-}$ o STAT6$^{-/-}$, respectivamente, se asoció con el tipo de respuesta inmune desencadenada, y no con la presencia de L2 *T. canis*.

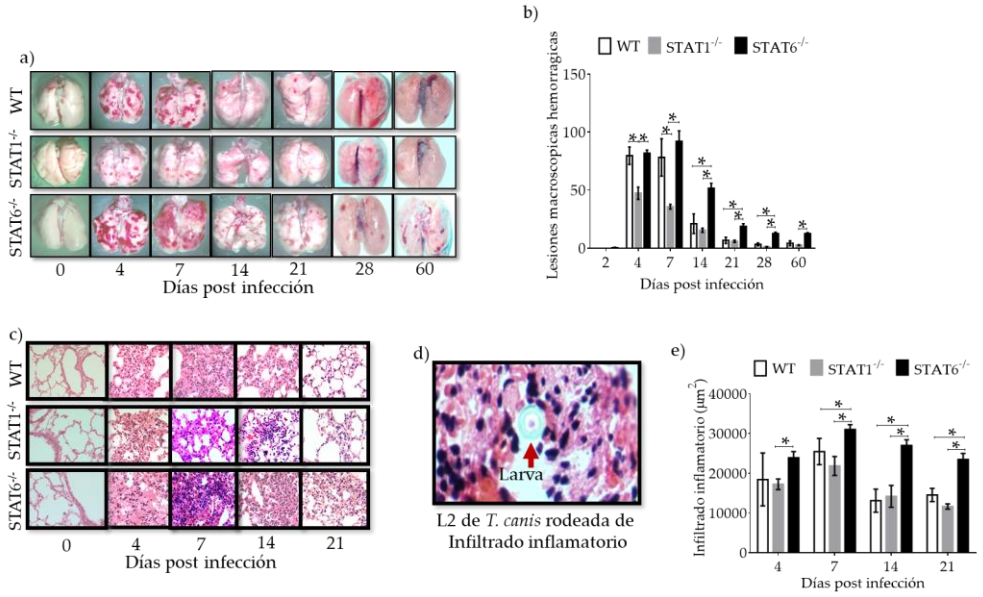

Figura 6. Lesiones hemorrágicas e infiltrado inflamatorio en tejido pulmonar. Los pulmones recolectados se lavaron con PBS, se fotografiaron de 0-60 dpi (a) y se contaron las lesiones hemorrágicas macroscópicas en ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$ (barras negras) (b). Secciones en parafina de 4 µm de grosor del tejido pulmonar fueron llevadas a cabo y se tiñeron con H&E, observándose infiltrado inflamatorio y lesiones hemorrágicas a la luz del microscopio óptico (objetivo 40x) a 0, 4, 7, 14 y 21 dpi en las tres cepas de ratones (c). Imagen representativa del L2 *T. canis* rodeada de infiltrado inflamatorio (objetivo 60x) (d). El infiltrado inflamatorio se cuantificó a partir de imágenes tomadas con un objetivo de 40x en microscopio óptico (e) en ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6^{-}

$^{/-}$ (barras negras). Los datos se muestran a partir de dos experimentos independientes como la media ± SEM (n = 6 por grupo); ANOVAde dos vías con post prueba de Tukey. *P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$ ratones en el mismo punto de infección.

6.3. Respuesta inmune asociada al daño pulmonar

Para determinar si una respuesta inmune inflamatoria o antiinflamatoria está asociada con la recuperación tardía del tejido pulmonar en ratones STAT6$^{-/-}$, citocinas y anticuerpos IgG específicos anti-*T. canis* se midieron en suero de sangre periférica. Las citocinas inflamatorias como IFN-γ (Figura 7a) y TNF-α (Figura 7b), así como IgG2a (Figura 7c), aumentaron en ratones STAT6$^{-/-}$ infectados con *T. canis* en comparación con ratones WT y STAT1$^{-/-}$ (Figura 7a-c). Las citocinas Th2, IL-4 (Figura 7d), IL-10 (Figura 7e) e IL-13 (Figura 7f) e IgG1 (Figura 7g), aumentaron en ratones WT y STAT1$^{-/-}$ pero no en ratones STAT6$^{-/-}$.

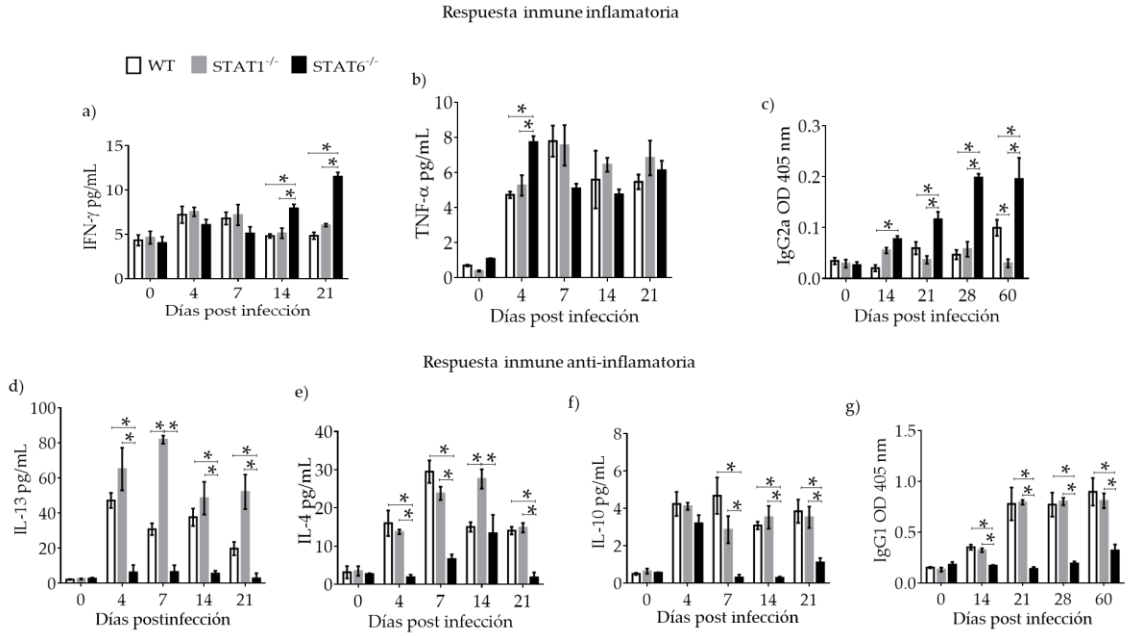

Figura 7. Respuesta inmune asociada con daño al tejido pulmonar durante la infección por *T. canis*. Citoquinas y anticuerpos de una respuesta inmune inflamatoria (IFN-γ (a), TNF-α (b), IgG2a (c)) y antiinflamatoria IL-4 (d), IL-13 (e), IL-10 (f), IgG1 (g) se midieron en suero a 0-60 dpi en ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$(barras negras). Los datos se muestran de dos experimentos independientes como la media ± SEM. Para las citocinas: (n = 4-6 por grupo); t no pareada con una post prueba Holm-Sidak. Para anticuerpos: (n = 6 por grupo); ANOVA de dos vías con una post prueba de Tukey. *P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6-/-, y STAT1$^{-/-}$ versus STAT6$^{-/-}$ ratones en el mismo punto de infección.

Para evaluar la respuesta inmune local, las citocinas de ambos perfiles inmunológicos se midieron por RT-PCR en fragmentos de tejido pulmonar. Los ratones STAT6$^{-/-}$ infectados con *T. canis* tenían una mayor expresión de IFN-γ (Figura 8a) y TNF-α (Figura 8b) al mismo tiempo que se observaron lesiones hemorrágicas macroscópicas en los pulmones. Por otro lado, los ratones STAT1$^{-/-}$ mostraron

una mayor expresión de IL-4 (Figura 8c) e IL-10 (Figura 8d y e), coincidiendo con el número reducido de lesiones macroscópicas en los pulmones. Estos resultados respaldan la hipótesis de que la recuperación retardada en el tejido pulmonar de ratones STAT6$^{-/-}$ podría estar asociada con un perfil proinflamatorio, mientras que una pronta recuperación en ratones STAT1$^{-/-}$ puede estar asociada con un perfil antiinflamatorio durante la infección de L2 de *T. canis*.

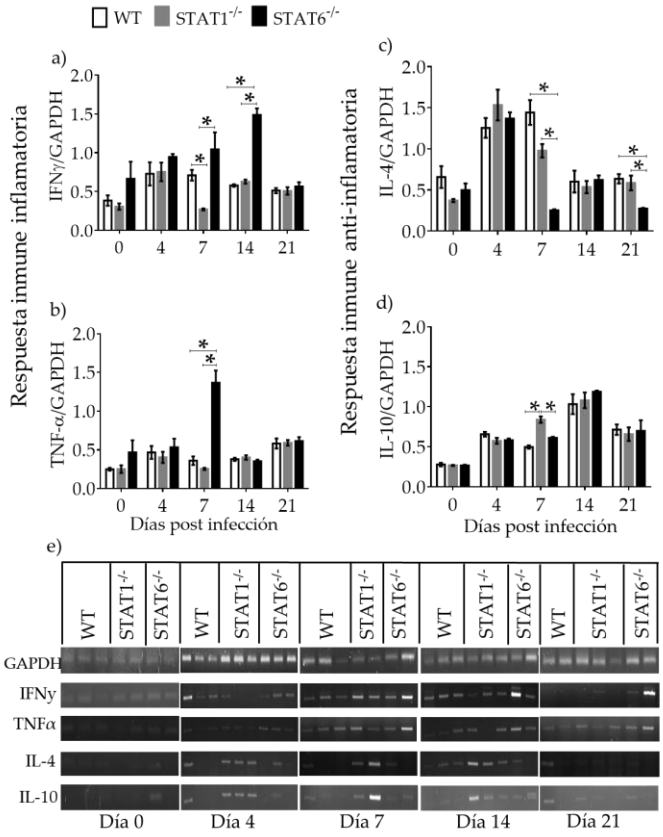

Figura 8. Respuesta inmune local asociada con daño de tejido pulmonar. La expresión del ARNm de IFN-γ (a), TNF-α (b), IL-4 (c) e IL-10 (d) se evaluó en tejido pulmonar de ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$ (barras negras) a 0, 4, 7, 14 y 21 dpi. Se muestra el gel de electroforesis de los respectivos genes y el control de GAPDH, como representativo de un experimento (e). Los datos se muestran de dos experimentos independientes como la media ± SEM (n = 5-6 por grupo); prueba t no pareada con post prueba Holm-Sidak; *P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$ ratones en el mismo punto de infección.

6.4. Marcadores de activación de los macrófagos M1 y M2 en tejido pulmonar durante la infección por T. canis.

IFN-γ es fundamental para la activación de macrófagos M1 y la expresión de iNOS [7, 9]. IL-4 e IL-13 son importantes para la diferenciación de macrófagos M2 y la expresión de Arg1, FIZZ1 e Ym1 [17]. Por lo tanto, el ARNm para estos marcadores M1 y M2 se midió en muestras de pulmón por RT-PCR. La expresión de ARNm de iNOS aumentó notablemente en ratones STAT6$^{-/-}$ infectados con *T. canis* (Figura 9a) dato que coincidió con la aparición de lesiones macroscópicas en los pulmones. En contraste, los ARNm de Arg1, FIZZ1 e Ym1 aumentaron en los pulmones de los ratones WT y STAT1$^{-/-}$ (Figura 9b-e).

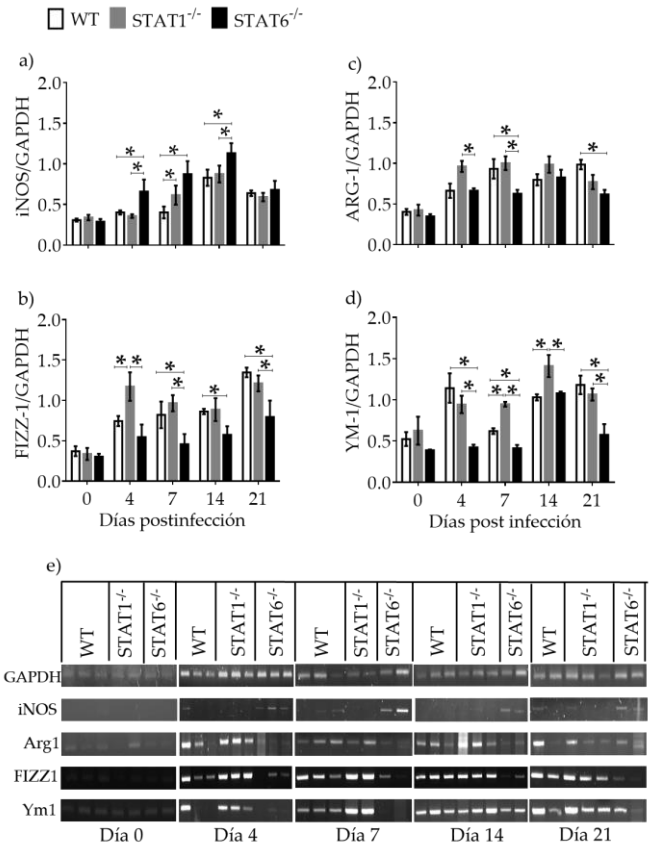

Figura 9. Marcadores de activación de macrófagos M1 y M2 en tejido pulmonar. La expresión de ARNm de iNOS (a), Arg1 (b), FIZZ1 (c) e Ym1 (d) se evaluó por RT-PCR en tejido pulmonar completo de ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$ (barras negras) en los días 0, 4, 7, 14 y 21 dpi. Se muestra un gel de electroforesis representativo de los diferentes genes mencionados anteriormente y su respectivo control de GAPDH de un experimento (e). Los datos de los gráficos se muestran a partir de dos experimentos independientes como la media ± SEM (n = 5-6 por grupo); prueba t no pareada con post prueba Holm-Sidak; * P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$ a los mismos tiempos de infección.

Para confirmar estos hallazgos, secciones de pulmón fueron evaluadas por inmunofluorescencia. Se sabe que la activación de STAT6 regula negativamente la expresión de iNOS en macrófagos [10]. De acuerdo con esto, iNOS se expresó desde los 4 dpi, y se mantuvo al menos hasta 14 dpi en ratones STAT6$^{-/-}$ (Figura 10a y b). En contraste, los ratones STAT1$^{-/-}$ presentaron una expresión reducida de iNOS, mientras que los ratones WT mostraron una expresión discreta de iNOS, particularmente a 4 dpi (Figura 10a y b). Con respecto a la expresión de Ym1, que se ha asociado constantemente con la reparación de tejidos [103], encontramos que su expresión temprana en los pulmones dependía en gran medida de la señalización de STAT6, dado que la expresión de Ym1 se redujo significativamente en los pulmones de ratones STAT6$^{-/-}$, mientras que células Ym1+ fueron abundantes en ratones WT y STAT1$^{-/-}$, especialmente a los 4 y 7 dpi (Figura 10c y d).

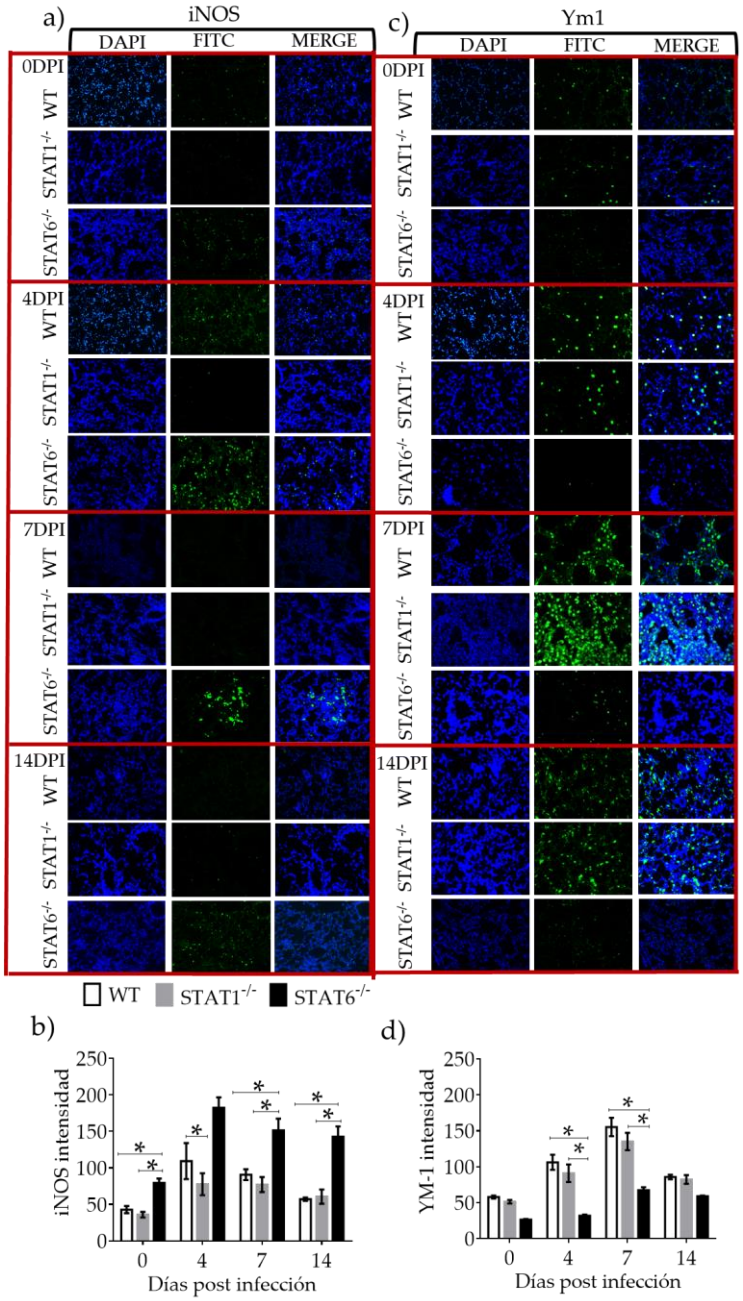

Figura 10. Evaluación de marcadores de activación M1 y M2 en tejido pulmonar por inmunofluorescencia. Secciones de pulmón de ratones WT, STAT1$^{-/-}$ y STAT6$^{-/-}$ a los 4, 7 y 14 dpi, marcados con el colorante de unión al ADN (DAPI) en azul e iNOS (a) o Ym1 (b) en verde. Cuantificación de la intensidad de fluorescencia de iNOS (c) y Ym1 (d) de las secciones de pulmón teñidas en b y c respectivamente, en ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$ (barras negras). Las fotografías fueron tomadas con un objetivo de 20x. Los datos se muestran a partir de dos experimentos independientes como la media ± SEM (n = 6 por grupo). ANOVA de dos vías con post prueba de Tukey. * P <0.05 comparando ratones WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$a los mismos tiempos de infección.

Para confirmar la presencia de macrófagos y determinar un probable papel en el daño o la reparación del tejido pulmonar, se evaluó por citometría de flujo la presencia de células F4/80-CD86 doble positivas sugestivas de activación M1 y células F4/80-IL4Rα y F4/80-MMR doblemente positivas para una activación M2 (Figura 11). Los ratones STAT6$^{-/-}$ mostraron un mayor porcentaje de células F4/80+CD86+ que no se observó en ratones WT o STAT1$^{-/-}$ (Figura 11b) a los 7 dpi. Sin

embargo, los ratones WT y STAT1$^{-/-}$ mostraron un mayor porcentaje de células F4/80+IL4Rα+ y F4/80+MMR+ (Figura 11c-d) después de la infección por *T. canis*.

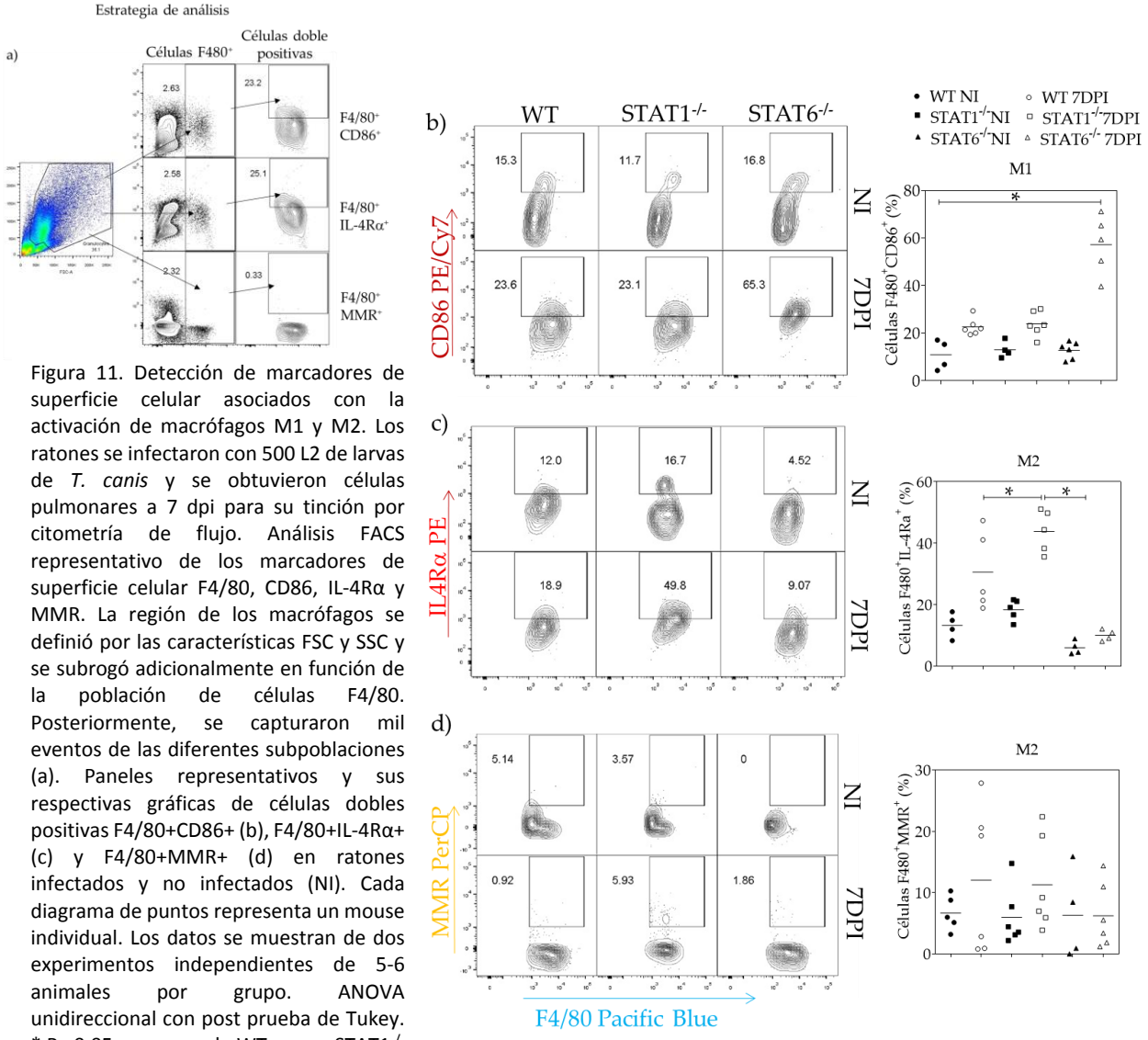

Figura 11. Detección de marcadores de superficie celular asociados con la activación de macrófagos M1 y M2. Los ratones se infectaron con 500 L2 de larvas de *T. canis* y se obtuvieron células pulmonares a 7 dpi para su tinción por citometría de flujo. Análisis FACS representativo de los marcadores de superficie celular F4/80, CD86, IL-4Rα y MMR. La región de los macrófagos se definió por las características FSC y SSC y se subrogó adicionalmente en función de la población de células F4/80. Posteriormente, se capturaron mil eventos de las diferentes subpoblaciones (a). Paneles representativos y sus respectivas gráficas de células dobles positivas F4/80+CD86+ (b), F4/80+IL-4Rα+ (c) y F4/80+MMR+ (d) en ratones infectados y no infectados (NI). Cada diagrama de puntos representa un mouse individual. Los datos se muestran de dos experimentos independientes de 5-6 animales por grupo. ANOVA unidireccional con post prueba de Tukey. * P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$ ratones.

En conjunto, estos datos sugieren que la deficiencia de STAT6 promueve la activación de macrófagos M1 y el daño al tejido pulmonar, mientras que la ausencia de STAT1 se asocia con la polarización de macrófagos M2 y la reparación temprana del tejido pulmonar durante la toxocariasis aguda.

6.5. Expresión diferencial de moléculas asociadas con regeneración tisular y fibrosis.

Preguntándonos si los mecanismos de reparación contribuyeron a la generación de fibrosis, se evaluaron factores asociados con el desarrollo de la fibrosis y la reparación de tejidos elastina, MMP-9 y TGF-β. La elastina, un factor de reparación de heridas, aumentó en todas las cepas de ratones a 21 dpi. Los ratones STAT6$^{-/-}$ infectados con *T. canis* tuvieron un aumento menor en elastina en comparación con los ratones WT y STAT1$^{-/-}$ a 4 dpi (Figura 12a y c). MMP9, una enzima que degrada la elastina, alcanzó el pico de expresión más alto a 14 dpi en todas las cepas de ratones. Sin embargo, los ratones STAT1$^{-/-}$ y STAT6$^{-/-}$ mostraron niveles más altos de MMP9 a 4 y 7 dpi (Figura 12b yd). Mientras que el ARNm de TGF-β, un factor asociado con la reparación del tejido producido por los macrófagos M2 [18] se expresó modestamente en ratones WT, se incrementó significativamente a diferentes dpi en ratones STAT1$^{-/-}$ y los ratones STAT6$^{-/-}$ no lograron mejorar la expresión de TGF-β en el tejido pulmonar (Figura 12e y f). Estas observaciones sugieren que ninguno de los grupos experimentales de ratones promueve una mayor fibrosis.

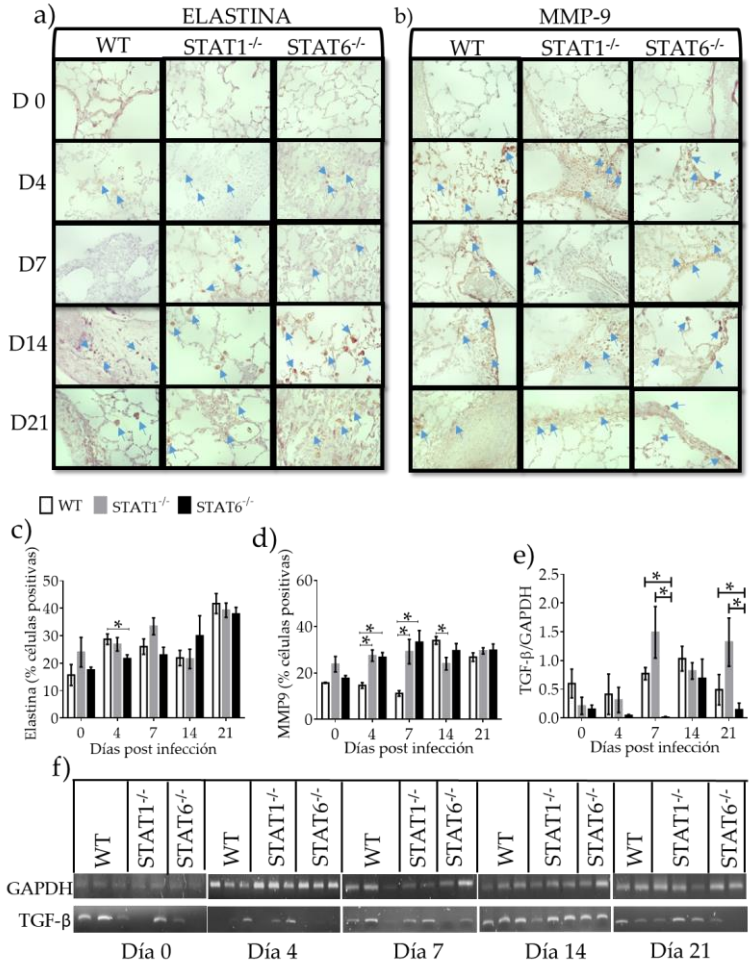

Figura 12. Moléculas asociadas con reparación de tejidos y fibrosis. Se procesaron secciones de tejido pulmonar en parafina para medir elastina (panel izquierdo) y MMP9 (panel derecho) por IHQ como marcadores de fibrosis. Se fotografiaron imágenes representativas de secciones de tejido pulmonar con el objetivo 60x (a, b), y se midió el porcentaje de células positivas para Elastina y MMP9 (marcas marrones señaladas por flechas) a diferentes dpi en ratones WT (barras blancas), STAT1$^{-/-}$ (barras grises) y STAT6$^{-/-}$ (barras negras) (c, d). La expresión de ARNm de TGF-β como marcador de reparación tisular se midió por RT-PCR (e) y se muestra su respectivo gel de electroforesis (f). Los datos son de dos experimentos independientes presentados como la media ± SEM (n = 5-6 por grupo); prueba de t no pareada con post prueba de Holm-Sidak; * P <0.05 comparando ratones WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$, y STAT1$^{-/-}$ versus STAT6$^{-/-}$ a los mismos tiempos de infección.

6.6. La depleción de macrófagos altera los procesos de la inflamación y reparación durante la toxocariasis aguda.

Para examinar si los macrófagos estaban involucrados en un proceso temprano de reparación del tejido pulmonar durante la infección aguda por L2 *T. canis*, los macrófagos fueron eliminados a través de la administración intratraqueal de liposomas con clodronato. Los ratones infectados WT y STAT1$^{-/-}$ tratados con PBS-liposomas mostraron lesiones hemorrágicas bien definidas, mientras que los que recibieron liposomas con clodronato mostraron lesiones graves y grandes que eran más difusas y extendidas a través de todo el parénquima pulmonar (Figura 13a). En ratones STAT6$^{-/-}$ infectados con *T. canis* tratados con liposomas PBS, las lesiones hemorrágicas eran obvias, numerosas y ampliamente distribuidas por todo el pulmón, en comparación con los ratones WT y STAT1$^{-/-}$ (Figura 13a). Por el contrario, los ratones STAT6$^{-/-}$ infectados tratados con liposomas con clodronato desarrollaron menos lesiones hemorrágicas en comparación con los ratones STAT6$^{-/-}$ infectados con *T. canis* y los ratones WT que recibieron liposomas con PBS. En particular, la administración de liposomas cargados de clodronato en ratones STAT6$^{-/-}$ redujo las lesiones hemorrágicas, y la apariencia de los pulmones fue similar a la de los ratones WT y STAT1$^{-/-}$ infectados con *T. canis* (Figura 13a), lo que sugiere que los macrófagos pueden mediar el control de la hemorragia y la inflamación causadas por el segundo estadio pulmonar de este helminto de una manera dependiente de STAT6. De acuerdo con estos hallazgos, el infiltrado inflamatorio se observó claramente afectado por la eliminación de los macrófagos. Por lo tanto, los ratones WT infectados con *T. canis* tratados con liposomas con clodronato mostraron infiltrados inflamatorios aumentados en comparación con los ratones WT infectados con *T. canis* que recibieron liposomas con PBS. De manera similar, la eliminación de macrófagos en ratones STAT1$^{-/-}$ infectados con *T. canis* dio como resultado un mayor infiltrado inflamatorio en los pulmones. En contraste, los ratones STAT6$^{-/-}$ infectados con *T. canis* tratados con liposomas con clodronato mostraron una reducción marcada en el infiltrado de células inflamatorias (Figura 13b y c). El análisis de citometría de flujo se realizó para evaluar la ausencia de los macrófagos. Los ratones que recibieron liposomas con clodronato tuvieron una reducción significativa en la población de células F4/80 + en las tres cepas infectadas de ratones, que oscila entre una reducción del 50-74% en el número de macrófagos pulmonares en comparación con los grupos control infectados y tratados con PBS-liposomas (Figura 13d).

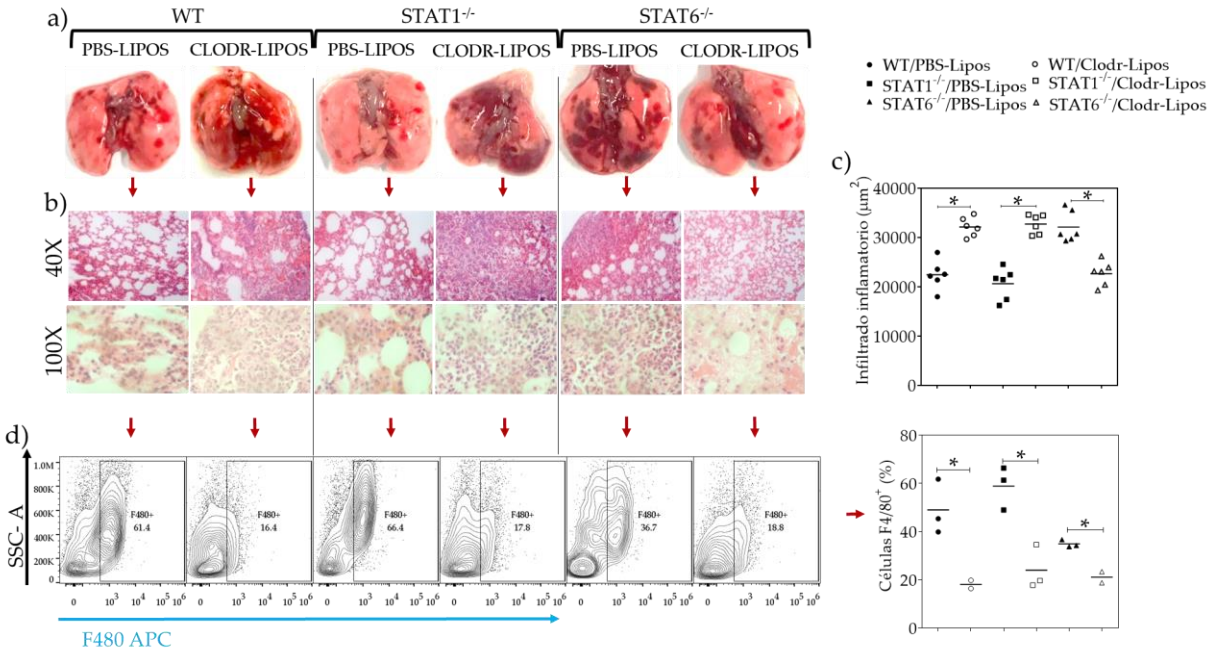

Figura 13. Eliminación de macrófagos pulmonares. Ratones WT, STAT1$^{-/-}$ y STAT6$^{-/-}$ fueron tratados con liposomas con PBS o liposomas con clodronato vía intratraqueal para eliminar los macrófagos, antes y después de ser infectados con 500 larvas de *T. canis* L2. Los animales fueron sacrificados a 4 dpi, y los pulmones fueron tomados y fotografiados (a). Se realizó tinción con H&E para obtener imágenes a 40x o 100x (b), y se midieron las áreas de infiltrado inflamatorio (e). Se recolectaron células de pulmón y por citometría de flujo se determinó la eficacia del tratamiento. Representación gráfica y sus respectivos porcentajes de células positivas para F4/80 de las tres cepas de ratones (c, d). * P <0.05 comparando WT versus STAT1$^{-/-}$ y STAT6$^{-/-}$ ratones.

7. DISCUSIÓN

El fenotipo de los macrófagos es determinado por las condiciones del microambiente y el medio local de citocinas [104]. Los macrófagos M1 tiene una fuerte actividad antimicrobiana y antitumoral, pueden generar daño tisular inducido por ROS, retrasar la regeneración tisular y la cicatrización de heridas [62, 105]. Por el contrario, el macrófago M2 antiinflamatorio regulador promueve la reparación mediante restitución de tejidos después del daño y además tiene un importante efecto antihelmíntico [55, 105-107].

La migración de helmintos a través del huésped hacia su órgano blanco puede causar un daño significativo en el tejido y, por lo tanto, es imperativo que el huésped sea capaz de iniciar una reparación rápida y efectiva del tejido. Los análisis de los sistemas de infecciones parasitarias en el modelo murino han arrojado un conocimiento considerable de las actividades celulares y moleculares responsables de la recuperación de tejidos después de la lesión [108, 109]. La movilización de los mecanismos de reparación es huesped-parásito específica, y diversas vías de regeneración tisular han sido estudiadas [110]. Sin embargo, hay una escasez de estudios para evaluar la reparación de tejidos en respuesta a la toxocariasis aguda [5, 6, 45], a pesar de que diferentes estudios han revelado que la respuesta inmune antiinflamatoria, a cargo de la activación de M2, es predominante durante la Infección por *T. canis* caracterizada por un incremento en la producción de citocinas como IL-13 e IL-4 y baja producción de IFN-γ y TNF-α [51-53, 111], valores que resultaron similares a los encontrados en el nuestro estudio.

En el presente estudio, se evaluó el papel de los macrófagos M1 y M2 en el proceso de reparación durante la etapa pulmonar de la infección por *T. canis* utilizando ratones STAT1$^{-/-}$ y STAT6$^{-/-}$. Según los hallazgos, la población de macrófagos M1 induce daño en los pulmones durante las primeras etapas de la migración de L2 de *T. canis*. En ratones STAT6$^{-/-}$, incluso cuando las larvas estaban casi ausentes de los pulmones en las etapas crónicas, el daño tisular persistió y se correlacionó con un aumento de iNOS indicativo de la presencia de células de tipo M1. De acuerdo con estas observaciones, la eliminación de los macrófagos en ratones STAT6$^{-/-}$ infectados con *T. canis* dio como resultado una reducción en el número de lesiones hemorrágicas en los pulmones y una mejor cicatrización. Juntos, estos datos sugieren fuertemente que los macrófagos M1 son un contribuyente importante al daño pulmonar durante las etapas tempranas y crónicas de la infección por L2 de *T. canis*. A pesar del daño producido en ausencia de STAT6, los mecanismos de reparación de tejidos asociados con una respuesta inmune antiinflamatoria no se suprimieron por completo.

Por lo tanto, los marcadores asociados con la regeneración tisular como Arg1, FIZZ1 e Ym1 eran detectables, al igual que el ARNm de TGF-β, mientras que el proceso regenerativo asociado con MMP9 y la expresión de elastina no se alteraron, dando como resultado procesos regenerativos retardados, aunque no del todo ausentes en los ratones STAT6$^{-/-}$ infectados. Estos datos están respaldados por un informe reciente de Sutherland y col; que muestra que las expresiones Ym1 y FIZZ1 no dependen completamente de la vía de señalización IL4R-α [112].

Otras vías de señalización, además de STAT6, están implicadas en la activación de macrófagos M2 y los mecanismos de reparación necesarios cuando los tejidos son dañados, los cuales involucran la señalización de Akt [113], STAT3 [114], IRF y PPARγ [8]. Además, alarminas epiteliales son secretadas seguida de la infección con parásitos nematodos para activar las ILC2 y conducir a una inmunidad tipo 2 [80, 81, 108], las cuales, vía IL 5 e IL 13 pueden movilizar los mecanismos de cicatrización con la capacidad para producir Arg1 [81] y FIZZ1 [115] para mantener la integridad del tejido. En consecuencia, en ratones STAT6$^{-/-}$ tratados con liposomas con clodronato, la depleción de los macrófagos redujo dicha población causante de la inflamación, promoviendo una activación más eficiente de todos los mecanismos de reparación y dando como resultado la reducción de las lesiones hemorrágicas. Resultados similares fueron reportados en ratones STAT6$^{-/-}$ infectados con *N. brasiliensis* en el que se observó un predominante perfil inflamatorio junto con la persistencia de la patología pulmonar [116]. Por lo tanto, el aumento de la inflamación conduce a una activación deficiente de macrófagos M2.

En nuestro modelo de infección con *T. canis*, evaluamos el papel de los macrófagos M2 en la reparación del tejido pulmonar al suprimir la respuesta inflamatoria a través de ratones STAT1$^{-/-}$. Observamos que el proceso regenerativo en esta cepa de ratón fue más rápido en comparación con los ratones STAT6$^{-/-}$ y WT. En ratones STAT1$^{-/-}$, fue evidente la activación de un perfil antiinflamatorio; la expresión de IL-13 aumentó; Arg1, FIZZ1 e Ym1 se expresaron de manera importante (o mostraron una tendencia a aumentar), y las células F4/80+IL-4Rα+ también se incrementaron significativamente y las células F4/80+CD86+ se mantuvieron a la baja. Finalmente, cuando los macrófagos fueron eliminados con liposomas con clodronato, se observó una patología pulmonar muy exacerbada. Estos datos en los ratones STAT1$^{-/-}$ confirman que los macrófagos M2 juegan un papel crucial en la regeneración del tejido pulmonar durante la infección por *T. canis*, y su ausencia compromete el proceso de reparación del tejido.

La respuesta inmune antiinflamatoria, comúnmente observada después de la infección con parásitos helmintos, regula la reparación del tejido mediante la actividad coordinada de células Th2, eosinófilos, mastocitos, basófilos, ILCs2 y macrófagos M2 [117, 118]. Sin embargo, cuando los procesos de reparación mediados por citocinas tipo 2 se vuelven crónicos, exuberantes o desregulados, pueden contribuir al desarrollo de fibrosis [118]. Diversos mecanismos que inducen la inflamación y la resolución de la fibrosis dependen del agente etiológico involucrado, el tropismo tisular y el tipo de respuesta inmune desencadenada en cada caso [119]. Otros factores asociados con la reparación de tejidos, como MMP2 y MMP9, pueden ser producidos por los macrófagos M2, están involucrados en la migración de queratinocitos y contribuyen a la eliminación de tejido no viable [120, 121]. En condiciones normales, la elastina es una proteína esencial del tejido conectivo que proporciona elasticidad y soporte a diversos órganos, incluidos los pulmones. Durante las patologías pulmonares, un aumento en la deposición de elastina puede causar fibrosis, y para evitar esto, MMP9 y MMP2 se activan para la degradación de proteínas y la reestructuración de la matriz extracelular (ECM) [122]. Aquí, observamos que MMP9 y elastina se producen en cantidades similares en las tres cepas [120]de ratones durante las primeras fases de la infección por *T. canis*. Por lo tanto, es factible que dicho fenómeno pueda ser una consecuencia del daño inducido por el helminto, que a su vez promueve la activación de los mecanismos necesarios para la reparación del tejido. Especulamos que el aumento de MMP9 contrarrestó el efecto de la deposición de elastina y colágeno, para evitar un aumento evidente de la fibrosis. Mientras que TGF-β expresada principalmente en ratones WT y STAT1$^{-/-}$ puede participar como un mecanismo regulador para facilitar la resolución de la respuesta proinflamatoria y fungir como impulsor de la reparación del tejido [55], por lo tanto, la ausencia de STAT6 compromete su expresión y la reparación de tejidos puede estar mediada por macrófagos M2.

Otro hallazgo importante fue que la respuesta inmune en ratones STAT6$^{-/-}$ condujo a la eliminación de las larvas de *T. canis*. Datos previos publicados por nuestro equipo de trabajo sobre la carga del parásito concluyeron que la producción de IgE no era esencial para la neutralización del parásito porque los ratones STAT6$^{-/-}$ no producían IgE pero tenían menor número de L2 de *T. canis* [24]. Un posible mecanismo subyacente a la resistencia a esta infección parasitaria en ratones STAT6$^{-/-}$ es el aumento de la activación de los macrófagos M1, lo que favorece la expresión de iNOS. Pocos estudios han propuesto un papel para el óxido nítrico (NO) en la resistencia contra los parásitos nematodos. En un estudio *in vitro*, Pfaff et al. demostró que las microfilarias de *L. sigmodontis* eran

vulnerable al ON. Sin embargo, ni la inhibición farmacéutica de la síntesis de óxido nítrico ni la eliminación de iNOS en ratones indujeron resistencia a *L. sigmodontis* circulante [123]. En un estudio más reciente en ratones iNOS$^{-/-}$ infectados con *Strongyloides venezuelensis* mostraron una mayor susceptibilidad a la infección [124]. Estos resultados demuestran que los mecanismos de protección o susceptibilidad definidos en un sistema o modelo no pueden asignarse simplemente a todas las interacciones helminto-huésped: diferencias bastante particulares pueden ser críticas para favorecer las respuestas inflamatorias iniciales que tienen un papel importante en inducir protección durante las infecciones por helmintos. Los datos de este estudio pueden interpretarse a favor de que iNOS contribuya en la resistencia de los ratones STAT6$^{-/-}$ a la infección con L2 de *T. canis.*

También pudimos observar que los ratones STAT1$^{-/-}$ tenían una menor carga parasitaria, similar a la de los ratones STAT6$^{-/-}$, con una evidente activación de los macrófagos M2. A diferencia de otras proteínas de la familia STAT, como STAT6 y STAT5, existen muy pocos reportes sobre cómo STAT1 participa en la modulación a la susceptibilidad o resistencia de las infecciones por helmintos. Es bien sabido que los macrófagos M2 dependientes de STAT6 juegan un papel importante durante las infecciones por helmintos en el control del daño y la eliminación de parásitos [17, 54, 125]. Una reciente publicación describió una participación para STAT1 durante la infección por *S. japonicum*, revelando un papel protector para miR16/4b en la inhibición de las células M1 mediante el bloqueo de STAT1, lo que resultó en la diferenciación a macrófagos M2 y resistencia parasitaria [22], este estudio respalda nuestros hallazgos en ratones STAT1$^{-/-}$ infectados con *T. canis*. Además, FIZZ1 producido por macrófagos M2 es una reconocida molécula, importante en la eliminación de *S. mansoni* o *N. brasiliensis* del intestino de ratones, efecto que se relacionó con una exacerbada respuesta inmune Th2 [126]. De hecho, en ausencia de STAT1, encontramos una marcada expresión de FIZZ-1 de forma aguda, por lo tanto, es posible que las larvas recién eclosionadas fueran expulsadas rápidamente del intestino y de los pulmones, debido a un aumento en la producción de moco induciendo una menor diseminación a través del flujo sanguíneo hacía otros órganos. En línea con esto, Filbey et al encontraron que las larvas migratorias de *N. brasiliensis* fueron eliminadas en los pulmones de ratones coinfectados con *H. polygyrus*, y la patología pulmonar asociada con la migración larvaria de *N. brasiliensis* se redujo debido a una robusta respuesta Th2 [126]. Finalmente, Esser-von Bieren et al sugirieron un nuevo mecanismo de activación M2 por anticuerpos,

independiente de IL-4Rα, que medía la actividad anti-helmintica y la alteración de los tejidos causada por la migración de las larvas de *H. polygyrus* [91].

Tradicionalmente, los eosinófilos son considerados como un importante mecanismo efector anti-helmintico [127-129], pero diversos estudios sugieren que dicho mecanismo no es tan simple; si bien la infección con *T. canis* se asocia con una respuesta eosinofílica, estas células pueden tener una capacidad limitada para matar el helminto y las larvas pueden escapar de manera eficiente de los eosinófilos, hecho que se ha demostrado en estudios in vitro [130-132]. En una revisión más reciente e interesante, se propuso un papel combinado para los macrófagos y los eosinófilos como factores clave en los mecanismos de formación de granulomas durante las infecciones por helmintos y en el equilibrio entre la reparación tisular y su eliminación [133]. Colectivamente, se propone un escenario en el que las respuestas inmunitarias inflamatorias y antiinflamatorias son importantes para mediar el daño tisular y la destrucción de helmintos. Por lo tanto, durante la infección por *T. canis*, los macrófagos M2 activados a través de STAT6 son células importantes para modular el daño del tejido pulmonar y conferir resistencia a la infección al inducir una potente respuesta inmunitaria antiinflamatoria, mientras que los macrófagos M1 activados a través de STAT1 promueven la patología pulmonar al mismo tiempo que la erradicación del parásito. Sin embargo, se necesita un estudio complementario y más extenso, que hable del papel de otras poblaciones de células junto con los macrófagos que pueden ayudar a mediar la eliminación de parásitos y la regeneración tisular durante la infección por L2 de *T. canis* como los eosinófilos o las células ILC2.

En resumen, nuestro trabajo extiende la importancia del papel de las vías STAT1 y STAT6 en la regeneración pulmonar durante la infección aguda por *T. canis* y destaca la importancia de los macrófagos M2 en la aceleración de la recuperación de tejidos durante la etapa pulmonar de esta infección.

8. CONCLUSIONES

- En ratones WT la susceptibilidad a la infección por *T. canis* ésta asociada con una respuesta inmune Th2.
- En ratones STAT6$^{-/-}$ la ausencia de dicha molécula promueve la resistencia a la infección, asociada con una respuesta inmune inflamatoria exacerbada mediada por M1
- Mientras que en ratones STAT1$^{-/-}$, también se promueve la resistencia a la infección, pero a través de una potente respuesta inmune antiinflamatoria mediada por la activación de M2.
- Se requiere un equilibrio entre ambas respuestas inmunes Th1-M1/Th2-M2 para la óptima neutralización y eliminación de las fases tisulares de *T. canis.*
- Los procesos de regeneración tisular en pulmón están fuertemente relacionados con la activación de macrófagos M2 y de sus factores de reparación FIZZ1, YM1, Arg1, TGF-β
- El daño a tejido pulmonar se encuentra asociado una respuesta inflamatoria mediada por la activación de M1 e iNOS.
- El equilibrio entre ambas poblaciones de macrófagos M1/M2 es importante para una regeneración armoniosa del tejido pulmonar.

9. REFERENCIAS

1. Ma, G., et al., *Human toxocariasis.* Lancet Infect Dis, 2018. **18**(1): p. e14-e24.
2. Rostami, A., et al., *Human toxocariasis - A look at a neglected disease through an epidemiological 'prism'.* Infect Genet Evol, 2019. **74**: p. 104002.
3. Nagy, D., et al., *Analysis of serum cytokine levels in children with chronic cough associated with Toxocara canis infection.* Parasite Immunol, 2012. **34**(12): p. 581-588.
4. Aghaei, S., et al., *Toxocara spp. infection and risk of childhood asthma: A systematic review and meta-analysis.* Acta Trop, 2018. **182**: p. 298-304.
5. Pinelli, E., et al., *Toxocara canis: effect of inoculum size on pulmonary pathology and cytokine expression in BALB/c mice.* Exp Parasitol, 2007. **115**(1): p. 76-82.
6. Zaia, M.G., et al., *Toxocara canis and the allergic process.* Mem Inst Oswaldo Cruz, 2015. **110**(6): p. 726-31.
7. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
8. Wang, N., H. Liang, and K. Zen, *Molecular mechanisms that influence the macrophage m1-m2 polarization balance.* Front Immunol, 2014. **5**: p. 614.
9. Li, C., et al., *Macrophage polarization and meta-inflammation.* Transl Res, 2018. **191**: p. 29-44.
10. Juhas, U., et al., *Different pathways of macrophage activation and polarization.* Postepy Hig Med Dosw (Online), 2015. **69**: p. 496-502.
11. Reich, N.C., *STATs get their move on.* JAKSTAT, 2013. **2**(4): p. e27080.
12. Ruckerl, D. and P.C. Cook, *Macrophages assemble! But do they need IL-4R during schistosomiasis?* Eur J Immunol, 2019. **49**(7): p. 996-1000.
13. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
14. Minutti, C.M., et al., *Local amplifiers of IL-4Ralpha-mediated macrophage activation promote repair in lung and liver.* Science, 2017. **356**(6342): p. 1076-1080.
15. Vannella, K.M. and T.A. Wynn, *Mechanisms of Organ Injury and Repair by Macrophages.* Annu Rev Physiol, 2017. **79**: p. 593-617.
16. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis.* Immunity, 2016. **44**(3): p. 450-462.
17. Kreider, T., et al., *Alternatively activated macrophages in helminth infections.* Curr Opin Immunol, 2007. **19**(4): p. 448-53.
18. Faz-Lopez, B., J. Morales-Montor, and L.I. Terrazas, *Role of Macrophages in the Repair Process during the Tissue Migrating and Resident Helminth Infections.* Biomed Res Int, 2016. **2016**: p. 8634603.
19. Anthony, R.M., et al., *Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites.* Nat Med, 2006. **12**(8): p. 955-60.
20. Li-Yin Hung, D.S., Taylor K. Oniskey, Jeremey Katzen, Noam A. Cohen, Andrew E. Vaughan, Wildaliz Nieves, Anatoly Urisman, Michael F. Beers, Matthew F. Krummel, and De'Broski R. Herbert, *Macrophages promote epithelial proliferation following infectious and non-infectious lung injury through a Trefoil factor 2-dependent mechanism.* Mucosal Immunol. , 2019. **12(1): 64–76.**
21. Jenkins, S.J., et al., *Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation.* Science, 2011. **332**(6035): p. 1284-8.

22. He, X., et al., *MicroR-146 blocks the activation of M1 macrophage by targeting signal transducer and activator of transcription 1 in hepatic schistosomiasis.* EBioMedicine, 2016. **13**: p. 339-347.
23. Nair, M.G. and D.R. Herbert, *Immune polarization by hookworms: taking cues from T helper type 2, type 2 innate lymphoid cells and alternatively activated macrophages.* Immunology, 2016. **148**(2): p. 115-24.
24. Faz-Lopez, B., et al., *Signal transducer and activator of transcription factor 6 signaling contributes to control host lung pathology but favors susceptibility against Toxocara canis infection.* Biomed Res Int, 2013. **2013**: p. 696343.
25. Murray, P.R., K.S. Rosenthal, and M.A. Pfaller, *Medical microbiology*. 8th edition. ed. x, 836 pages.
26. Chen, J., et al., *Toxocariasis: a silent threat with a progressive public health impact.* Infect Dis Poverty, 2018. **7**(1): p. 59.
27. Romero Nunez, C., et al., *Prevalence and risk factors associated with Toxocara canis infection in children.* ScientificWorldJournal, 2013. **2013**: p. 572089.
28. El-Tras, W.F., H.R. Holt, and A.A. Tayel, *Risk of Toxocara canis eggs in stray and domestic dog hair in Egypt.* Vet Parasitol, 2011. **178**(3-4): p. 319-23.
29. Finsterer, J. and H. Auer, *Parasitoses of the human central nervous system.* J Helminthol, 2013. **87**(3): p. 257-70.
30. Rubinsky-Elefant, G., et al., *Human toxocariasis: diagnosis, worldwide seroprevalences and clinical expression of the systemic and ocular forms.* Ann Trop Med Parasitol, 2010. **104**(1): p. 3-23.
31. Glickman, L.T. and P.M. Schantz, *Epidemiology and pathogenesis of zoonotic toxocariasis.* Epidemiol Rev, 1981. **3**: p. 230-50.
32. Overgaauw, P.A. and F. van Knapen, *Veterinary and public health aspects of Toxocara spp.* Vet Parasitol, 2013. **193**(4): p. 398-403.
33. Macpherson, C.N., *The epidemiology and public health importance of toxocariasis: a zoonosis of global importance.* Int J Parasitol, 2013. **43**(12-13): p. 999-1008.
34. Nicoletti, A., *Toxocariasis.* Handb Clin Neurol, 2013. **114**: p. 217-28.
35. Shoop, W.L., *Vertical transmission of helminths: Hypobiosis and amphiparatenesis.* Parasitol Today, 1991. **7**(2): p. 51-4.
36. Magnaval, J.F., et al., *Highlights of human toxocariasis.* Korean J Parasitol, 2001. **39**(1): p. 1-11.
37. Winders, W.T. and L. Menkin-Smith, *Toxocara Canis (Visceral Larva Migrans, Toxocariasis)*, in *StatPearls*2019: Treasure Island (FL).
38. Zhang, T., et al., *Ocular Toxocariasis: Long-Term Follow-Up and Prognosis of Patients following Vitrectomy.* Ocul Immunol Inflamm, 2019: p. 1-7.
39. Daoudi, C., et al., *[Ocular toxocariasis: report of two cases and review of the literature].* Pan Afr Med J, 2014. **17**: p. 71.
40. Vidal, J.E., J. Sztajnbok, and A.C. Seguro, *Eosinophilic meningoencephalitis due to Toxocara canis: case report and review of the literature.* Am J Trop Med Hyg, 2003. **69**(3): p. 341-3.
41. Lee, R.M., et al., *Toxocariasis in North America: a systematic review.* PLoS Negl Trop Dis, 2014. **8**(8): p. e3116.
42. Cortés NN, N.C., Guiliana BG, García PA, Cárdenas RH., *Presence of anti-Toxocara canis antibodies and risk factors in children from the Amecameca and Chalco regions of México.* BMC Pediatr., 2015. **30;15:65.** .

43. Pinelli, E., et al., *Infection with the roundworm Toxocara canis leads to exacerbation of experimental allergic airway inflammation.* Clin Exp Allergy, 2008. **38**(4): p. 649-58.
44. Pinelli, E., et al., *Persistent airway hyper-responsiveness and inflammation in Toxocara canis-infected BALB/c mice.* Clin Exp Allergy, 2005. **35**(6): p. 826-32.
45. Maizels, R.M., *Toxocara canis: molecular basis of immune recognition and evasion.* Vet Parasitol, 2013. **193**(4): p. 365-74.
46. Bashi, T., et al., *The mechanisms behind helminth's immunomodulation in autoimmunity.* Autoimmun Rev, 2015. **14**(2): p. 98-104.
47. Cardillo, N., et al., *Experimental infection with Toxocara cati in BALB/c mice, migratory behaviour and pathological changes.* Zoonoses Public Health, 2009. **56**(4): p. 198-205.
48. Schoenardie, E.R., et al., *Determination of IgG avidity in BALB/c mice experimentally infected with Toxocara canis.* Rev Bras Parasitol Vet, 2014. **23**(3): p. 403-6.
49. Schoenardie, E.R., et al., *Vertical transmission of Toxocara canis in successive generations of mice.* Rev Bras Parasitol Vet, 2013. **22**(4): p. 623-6.
50. Pecinali, N.R., et al., *Influence of murine Toxocara canis infection on plasma and bronchoalveolar lavage fluid eosinophil numbers and its correlation with cytokine levels.* Vet Parasitol, 2005. **134**(1-2): p. 121-30.
51. Resende, N.M., et al., *New insights into the immunopathology of early Toxocara canis infection in mice.* Parasit Vectors, 2015. **8**: p. 354.
52. Frantz, F.G., et al., *The immune response to toxocariasis does not modify susceptibility to Mycobacterium tuberculosis infection in BALB/c mice.* Am J Trop Med Hyg, 2007. **77**(4): p. 691-8.
53. Pilarczyk, B., et al., *Selenium supplementation enhances the protective response to Toxocara canis larvae in mice.* Parasite Immunol, 2008. **30**(8): p. 394-402.
54. Bai, X., et al., *[Alternatively activated macrophages in helminth infections].* Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi, 2011. **29**(3): p. 219-23.
55. Noel, W., et al., *Alternatively activated macrophages during parasite infections.* Trends Parasitol, 2004. **20**(3): p. 126-33.
56. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-55.
57. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and functions of tissue macrophages.* Immunity, 2014. **41**(1): p. 21-35.
58. Sica, A., et al., *Macrophage polarization in pathology.* Cell Mol Life Sci, 2015. **72**(21): p. 4111-26.
59. Benoit, M., B. Desnues, and J.L. Mege, *Macrophage polarization in bacterial infections.* J Immunol, 2008. **181**(6): p. 3733-9.
60. Das, A., et al., *Monocyte and macrophage plasticity in tissue repair and regeneration.* Am J Pathol, 2015. **185**(10): p. 2596-606.
61. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas.* J Clin Invest, 2012. **122**(3): p. 787-95.
62. Shapouri-Moghaddam, A., et al., *Macrophage plasticity, polarization, and function in health and disease.* J Cell Physiol, 2018. **233**(9): p. 6425-6440.
63. Biswas, S.K., et al., *Macrophage polarization and plasticity in health and disease.* Immunol Res, 2012. **53**(1-3): p. 11-24.
64. Cassetta, L., E. Cassol, and G. Poli, *Macrophage polarization in health and disease.* ScientificWorldJournal, 2011. **11**: p. 2391-402.

65. Herbert, D.R., et al., *Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology.* Immunity, 2004. **20**(5): p. 623-35.
66. Nair, M.G., et al., *Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells.* Infect Immun, 2005. **73**(1): p. 385-94.
67. Braga, T.T., J.S. Agudelo, and N.O. Camara, *Macrophages During the Fibrotic Process: M2 as Friend and Foe.* Front Immunol, 2015. **6**: p. 602.
68. Murray, P.J. and T.A. Wynn, *Obstacles and opportunities for understanding macrophage polarization.* J Leukoc Biol, 2011. **89**(4): p. 557-63.
69. Abroun, S., et al., *STATs: An Old Story, Yet Mesmerizing.* Cell J, 2015. **17**(3): p. 395-411.
70. Zundler, S. and M.F. Neurath, *Integrating Immunologic Signaling Networks: The JAK/STAT Pathway in Colitis and Colitis-Associated Cancer.* Vaccines (Basel), 2016. **4**(1).
71. Villarino, A.V., et al., *Mechanisms of Jak/STAT signaling in immunity and disease.* J Immunol, 2015. **194**(1): p. 21-7.
72. Schwartz, D.M., et al., *Type I/II cytokines, JAKs, and new strategies for treating autoimmune diseases.* Nat Rev Rheumatol, 2016. **12**(1): p. 25-36.
73. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. **11**(11): p. 723-37.
74. Date, D., et al., *Kruppel-like transcription factor 6 regulates inflammatory macrophage polarization.* J Biol Chem, 2014. **289**(15): p. 10318-29.
75. Lawrence, T. and G. Natoli, *Transcriptional regulation of macrophage polarization: enabling diversity with identity.* Nat Rev Immunol, 2011. **11**(11): p. 750-61.
76. Chen, F., et al., *An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection.* Nat Med, 2012. **18**(2): p. 260-6.
77. Pesce, J.T., et al., *Retnla (relmalpha/fizz1) suppresses helminth-induced Th2-type immunity.* PLoS Pathog, 2009. **5**(4): p. e1000393.
78. Sutherland, T.E., et al., *Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage.* Nat Immunol, 2014. **15**(12): p. 1116-25.
79. Chen, F., et al., *Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion.* Nat Immunol, 2014. **15**(10): p. 938-46.
80. Turner, J.E., et al., *IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation.* J Exp Med, 2013. **210**(13): p. 2951-65.
81. Bando, J.K., et al., *Type 2 innate lymphoid cells constitutively express arginase-I in the naive and inflamed lung.* J Leukoc Biol, 2013. **94**(5): p. 877-84.
82. Vannella, K.M., et al., *Incomplete deletion of IL-4Ralpha by LysM(Cre) reveals distinct subsets of M2 macrophages controlling inflammation and fibrosis in chronic schistosomiasis.* PLoS Pathog, 2014. **10**(9): p. e1004372.
83. Chiaramonte, M.G., et al., *IL-13 Is a Key Regulatory Cytokine for Th2 Cell-Mediated Pulmonary Granuloma Formation and IgE Responses Induced by Schistosoma mansoni Eggs.* The Journal of Immunology, 1999. **162**(2): p. 920-930.
84. Kurakula, K., et al., *LIM-only protein FHL2 is a positive regulator of liver X receptors in smooth muscle cells involved in lipid homeostasis.* Mol Cell Biol, 2015. **35**(1): p. 52-62.
85. Borthwick, L.A., et al., *Macrophages are critical to the maintenance of IL-13-dependent lung inflammation and fibrosis.* Mucosal Immunol, 2016. **9**(1): p. 38-55.

86. Herbert, D.R., et al., *Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis.* J Immunol, 2010. **184**(11): p. 6438-46.
87. Tundup, S., et al., *CD14 influences host immune responses and alternative activation of macrophages during Schistosoma mansoni infection.* Infect Immun, 2014. **82**(8): p. 3240-51.
88. Edukulla, R., et al., *Th2 Cytokines Augment IL-31/IL-31RA Interactions via STAT6-dependent IL-31RA Expression.* J Biol Chem, 2015. **290**(21): p. 13510-20.
89. Rani, R., et al., *IFN-gamma-driven IDO production from macrophages protects IL-4Ralpha-deficient mice against lethality during Schistosoma mansoni infection.* Am J Pathol, 2012. **180**(5): p. 2001-8.
90. Nascimento, M., et al., *Ly6Chi monocyte recruitment is responsible for Th2 associated host-protective macrophage accumulation in liver inflammation due to schistosomiasis.* PLoS Pathog, 2014. **10**(8): p. e1004282.
91. Esser-von Bieren, J., et al., *Antibodies trap tissue migrating helminth larvae and prevent tissue damage by driving IL-4Ralpha-independent alternative differentiation of macrophages.* PLoS Pathog, 2013. **9**(11): p. e1003771.
92. Mylonas, K.J., et al., *The adult murine heart has a sparse, phagocytically active macrophage population that expands through monocyte recruitment and adopts an 'M2' phenotype in response to Th2 immunologic challenge.* Immunobiology, 2015. **220**(7): p. 924-33.
93. Okada, H., et al., *Effect of nematode Trichinella infection on glucose tolerance and status of macrophage in obese mice.* Endocr J, 2013. **60**(11): p. 1241-9.
94. Du, L., et al., *The protective effect of the recombinant 53-kDa protein of Trichinella spiralis on experimental colitis in mice.* Dig Dis Sci, 2011. **56**(10): p. 2810-7.
95. Leon-Cabrera, S., et al., *Extraintestinal helminth infection reduces the development of colitis-associated tumorigenesis.* Int J Biol Sci, 2014. **10**(9): p. 948-56.
96. Ledesma-Soto, Y., et al., *Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins.* Biomed Res Int, 2015. **2015**: p. 563425.
97. Reyes, J.L., et al., *Taenia crassiceps infection abrogates experimental autoimmune encephalomyelitis.* Cell Immunol, 2011. **267**(2): p. 77-87.
98. Bowcutt, R., et al., *Arginase-1-expressing macrophages are dispensable for resistance to infection with the gastrointestinal helminth Trichuris muris.* Parasite Immunol, 2011. **33**(7): p. 411-20.
99. Nagy, D., et al., *Analysis of serum cytokine levels in children with chronic cough associated with Toxocara canis infection.* Parasite Immunol, 2012. **34**(12): p. 581-8.
100. Bahnea, R.G., et al., *[Toxocariasis respiratory manifestations in cases hospitalized in the Paediatric Diseases Clinic of Iasi, between 2005-2008].* Rev Med Chir Soc Med Nat Iasi, 2009. **113**(4): p. 1099-101.
101. Malheiro, A., et al., *pcDNA-IL-12 vaccination blocks eosinophilic inflammation but not airway hyperresponsiveness following murine Toxocara canis infection.* Vaccine, 2008. **26**(3): p. 305-15.
102. Watthanakulpanich, D., et al., *Application of Toxocara canis excretory-secretory antigens and IgG subclass antibodies (IgG1-4) in serodiagnostic assays of human toxocariasis.* Acta Trop, 2008. **106**(2): p. 90-5.
103. Ikeda, N., et al., *Emergence of immunoregulatory Ym1(+)Ly6C(hi) monocytes during recovery phase of tissue injury.* Sci Immunol, 2018. **3**(28).

104. Franken, L., M. Schiwon, and C. Kurts, *Macrophages: sentinels and regulators of the immune system.* Cell Microbiol, 2016. **18**(4): p. 475-87.
105. Gordon, S., A. Pluddemann, and F. Martinez Estrada, *Macrophage heterogeneity in tissues: phenotypic diversity and functions.* Immunol Rev, 2014. **262**(1): p. 36-55.
106. Porta, C., et al., *Molecular and epigenetic basis of macrophage polarized activation.* Semin Immunol, 2015. **27**(4): p. 237-48.
107. Schultze, J.L. and S.V. Schmidt, *Molecular features of macrophage activation.* Semin Immunol, 2015. **27**(6): p. 416-23.
108. Gause, W.C., T.A. Wynn, and J.E. Allen, *Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths.* Nat Rev Immunol, 2013. **13**(8): p. 607-14.
109. Allen, J.E. and T.E. Sutherland, *Host protective roles of type 2 immunity: parasite killing and tissue repair, flip sides of the same coin.* Semin Immunol, 2014. **26**(4): p. 329-40.
110. Aranzamendi, C., L. Sofronic-Milosavljevic, and E. Pinelli, *Helminths: Immunoregulation and Inflammatory Diseases-Which Side Are Trichinella spp. and Toxocara spp. on?* J Parasitol Res, 2013. **2013**: p. 329438.
111. Waindok, P. and C. Strube, *Neuroinvasion of Toxocara canis- and T. cati-larvae mediates dynamic changes in brain cytokine and chemokine profile.* J Neuroinflammation, 2019. **16**(1): p. 147.
112. Sutherland, T.E., et al., *Ym1 induces RELMalpha and rescues IL-4Ralpha deficiency in lung repair during nematode infection.* PLoS Pathog, 2018. **14**(11): p. e1007423.
113. Vergadi, E., et al., *Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization.* J Immunol, 2017. **198**(3): p. 1006-1014.
114. O'Farrell, A.M., et al., *IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways.* EMBO J, 1998. **17**(4): p. 1006-18.
115. Sonnenberg, G.F. and D. Artis, *Innate lymphoid cells in the initiation, regulation and resolution of inflammation.* Nat Med, 2015. **21**(7): p. 698-708.
116. Reece, J.J., et al., *Hookworm-induced persistent changes to the immunological environment of the lung.* Infect Immun, 2008. **76**(8): p. 3511-24.
117. Loser, S., K.A. Smith, and R.M. Maizels, *Innate Lymphoid Cells in Helminth Infections-Obligatory or Accessory?* Front Immunol, 2019. **10**: p. 620.
118. Gieseck, R.L., 3rd, M.S. Wilson, and T.A. Wynn, *Type 2 immunity in tissue repair and fibrosis.* Nat Rev Immunol, 2018. **18**(1): p. 62-76.
119. Gazzinelli-Guimaraes, P.H. and T.B. Nutman, *Helminth parasites and immune regulation.* F1000Res, 2018. **7**.
120. Soldano, S., et al., *Correction: Alternatively Activated (M2) Macrophage Phenotype Is Inducible by Endothelin-1 in Cultured Human Macrophages.* PLoS One, 2017. **12**(3): p. e0175238.
121. Jimenez, P.A. and S.E. Jimenez, *Tissue and cellular approaches to wound repair.* Am J Surg, 2004. **187**(5A): p. 56S-64S.
122. Reddel, C.J., A.S. Weiss, and J.K. Burgess, *Elastin in asthma.* Pulm Pharmacol Ther, 2012. **25**(2): p. 144-53.
123. Pfaff, A.W., et al., *The role of nitric oxide in the innate resistance to microfilariae of Litomosoides sigmodontis in mice.* Parasite Immunol, 2000. **22**(8): p. 397-405.
124. Rodrigues, R.M., et al., *Inducible nitric oxide synthase controls experimental Strongyloides infection.* Parasite Immunol, 2018. **40**(10): p. e12576.

125. Barron, L. and T.A. Wynn, *Macrophage activation governs schistosomiasis-induced inflammation and fibrosis.* Eur J Immunol, 2011. **41**(9): p. 2509-14.
126. Filbey, K.J., et al., *Intestinal helminth infection promotes IL-5- and CD4(+) T cell-dependent immunity in the lung against migrating parasites.* Mucosal Immunol, 2019. **12**(2): p. 352-362.
127. Masure, D., et al., *A role for eosinophils in the intestinal immunity against infective Ascaris suum larvae.* PLoS Negl Trop Dis, 2013. **7**(3): p. e2138.
128. Galioto, A.M., et al., *Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval strongyloides stercoralis in mice.* Infect Immun, 2006. **74**(10): p. 5730-8.
129. Holmes, D.A., et al., *Dusp5 negatively regulates IL-33-mediated eosinophil survival and function.* EMBO J, 2015. **34**(2): p. 218-35.
130. Rockey, J.H., et al., *In vitro interaction of eosinophils from ascarid-infected eyes with Ascaris suum and Toxocara canis larvae.* Invest Ophthalmol Vis Sci, 1983. **24**(10): p. 1346-57.
131. Takamoto, M., et al., *Eosinophilia, parasite burden and lung damage in Toxocara canis infection in C57Bl/6 mice genetically deficient in IL-5.* Immunology, 1997. **90**(4): p. 511-7.
132. Rodolpho, J.M.A., et al., *Robust Phenotypic Activation of Eosinophils during Experimental Toxocara canis Infection.* Front Immunol, 2018. **9**: p. 64.
133. Ariyaratne, A. and C.A.M. Finney, *Eosinophils and Macrophages within the Th2-Induced Granuloma: Balancing Killing and Healing in a Tight Space.* Infect Immun, 2019. **87**(10).

10. ANEXOS

10.1. Artículos publicados

- Berenice Faz-López, Héctor Mayoral-Reyes, Rogelio Hernández-Pando, Pablo Martínez-Labat, Derek M. McKay, Itzel Medina-Andrade, Jonadab E. Olguín and Luis I. Terrazas; A dual role for macrophages in modulating lung tissue damage/repair during L2 *Toxocara canis* infection. Pathogens. 2019 Dec 2; 8(4). pii: E280. doi: 10.3390/pathogens8040280.
- C. E. Matisz, B Faz-López, E. Thomson, A. Al Rajabi, F. Lopes, L. I. Terrazas, A. Wang, K. A. Sharkey, D. M. McKay; Suppression of colitis by adoptive transfer of helminth antigen-treated dendritic cells requires interleukin-4 receptor-α signaling. Scientific Report. 2017 17; 7: 40631.doi: 10.1038 / srep40631.
- Faz-López B, Morales-Montor J, Terrazas LI; Role of macrophages in the repair process during tissue migration and resident helminth infections. Biomed Res Int. 2016; 2016: 8634603. doi: 10.1155 / 2016/8634603.
- Faz-López B, Ledesma-Soto Y, Romero-Sánchez Y, Calleja E, Martínez-Labat P, Terrazas LI; Signal Transducer and Activator of Transcription (STAT) -6 signaling, contributes to control host lung pathology but favors susceptibility against *Toxocara canis* infection. Biomed Res Int. 2013; 2013: 696343. doi: 10.1155 / 2013/696343.

pathogens

MDPI

Article

A Dual Role for Macrophages in Modulating Lung Tissue Damage/Repair during L2 *Toxocara canis* Infection

Berenice Faz-López [1], Héctor Mayoral-Reyes [2,3], Rogelio Hernández-Pando [3], Pablo Martínez-Labat [4], Derek M. McKay [5], Itzel Medina-Andrade [6], Jonadab E. Olguin [6] and Luis I. Terrazas [1,6,*]

1 Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), Tlalnepantla 54090, MEX, México; berefaz@comunidad.unam.mx
2 Servicio de Medicina Interna, Hospital General de México Dr. Eduardo Liceaga, Ciudad de México 06720, México; hectormayoral@comunidad.unam.mx
3 Departamento de Patología, Sección de Patología Experimental, Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán, Ciudad de México 14000, México; rogelio.hernandezp@incmnsz.mx
4 Laboratorio de Parasitología, Facultad de Estudios Superiores Cuautitlán, UNAM, Cuautitlán I 54716, MEX, México; jpmlabat@servidor.unam.mx
5 Gastrointestinal Research Group and Inflammation Research Network, Department of Physiology and Pharmacology, Calvin, Joan and Phoebe Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada; dmckay@ucalgary.ca
6 Laboratorio Nacional en Salud, Facultad de Estudios Superiores Iztacala, UNAM, Tlalnepantla 54090, MEX, México; xoxo_pixar@comunidad.unam.mx (I.M.-A.); e.olguin@comunidad.unam.mx (J.E.O.)
* Correspondence: literrazas@unam.mx; Tel.: +52-55-5623-1333 (ext. 39773)

Received: 18 October 2019; Accepted: 26 November 2019; Published: 2 December 2019

Abstract: Macrophages that are classically activated (M1) through the IFN-γ/STAT1 signaling pathway have a major role in mediating inflammation during microbial and parasitic infections. In some cases, unregulated inflammation induces tissue damage. In helminth infections, alternatively activated macrophages (M2), whose activation occurs mainly via the IL-4/STAT6 pathway, have a major role in mediating protection against excessive inflammation, and has been associated with both tissue repair and parasite clearance. During the lung migratory stage of *Toxocara canis*, the roles of M1 and M2 macrophages in tissue repair remain unknown. To assess this, we orally infected wild-type (WT) and STAT1 and STAT6-deficient mice ($STAT1^{-/-}$ and $STAT6^{-/-}$) with L2 *T. canis*, and evaluated the role of M1 or M2 macrophages in lung pathology. The absence of STAT1 favored an M2 activation pattern with Arg1, FIZZ1, and Ym1 expression, which resulted in parasite resistance and lung tissue repair. In contrast, the absence of STAT6 induced M1 activation and iNOS expression, which helped control parasitic infection but generated increased inflammation and lung pathology. Next, macrophages were depleted by intratracheally inoculating mice with clodronate-loaded liposomes. We found a significant reduction in alveolar macrophages that was associated with higher lung pathology in both WT and $STAT1^{-/-}$ mice; in contrast, $STAT6^{-/-}$ mice receiving clodronate-liposomes displayed less tissue damage, indicating critical roles of both macrophage phenotypes in lung pathology and tissue repair. Therefore, a proper balance between inflammatory and anti-inflammatory responses during *T. canis* infection is necessary to limit lung pathology and favor lung healing.

Keywords: STAT1; STAT6; M1-M2 macrophages; tissue damage-repair; parasite resistance

1. Introduction

Toxocariasis is a neglected disease despite being broadly distributed, and there has been limited attention given to prevention, treatment, and disease surveillance [1]. Adult *Toxocara canis* worms live in the small intestine of dogs, which excrete eggs in the feces, polluting the environment [2]. Once in the small intestine of intermediate hosts (it can be paratenic hosts, such as humans or mice), larvae penetrate the gut wall and migrate to many organs, including the lung, liver, muscles, and central nervous system, resulting in significant pathology. Infection in the lung with *T. canis* L2 larvae can cause an asthma-type condition in children [3,4]; airway hyperresponsiveness, pulmonary inflammation, and increased levels of IgE can persist for months in mice experimentally-infected with *T. canis* [5,6]. Thus, assessing the inflammatory response, including defining roles for cells such as classically activated macrophages (M1) in the pathology following infection with *T. canis* is critical to a better understanding of the disease and the development of targeted therapies.

M1 macrophages are primarily activated via interferon (IFN)-γ through signaling pathways, such as signal transducer and activator of transcription 1 (STAT1), and interferon regulatory factors (IRF) 1 and 8, which allow expansion of the inflammatory immune response [7,8]. M1 macrophages produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, and IL-23 and nitric oxide (NO) through the enzyme inducible nitric oxide synthase (iNOS) [9], and have high expression of the co-stimulatory CD80 and CD86 molecules [10]. On the other hand, during helminth-infection, there are both potent type 2 and immunoregulatory networks that can limit tissue damage caused by the parasite (or the immune response against them). A cellular hallmark of anti-parasitic type 2 immunity is alternatively activated macrophages (M2), activated by interleukin (IL)-4 and IL-13 through the IL-4 receptor alpha (IL-4Rα), and mobilization of STAT6, c-Myc, and IRF4 [7,11–13]. Thus, M2 activation promotes control of helminth infection and tissue repair [14,15]. STAT6 regulates many of the genes associated with mouse M2 macrophages, including arginase 1 (Arg1), resistin-like-α (Retnlα, Relmα or FIZZ1), chitinase 3-like 3 (Chi3l3 or Ym1), and macrophage mannose receptor (MMR) or CD206. M2 macrophages have the capacity to block iNOS, counteracting tissue damage caused by M1 macrophages [8,10]. Furthermore, M2 macrophages activated via STAT6 are an important source of chemokines, cytokines, elastin, matrix metalloproteinases (MMPs), and other mediators that drive cellular responses following tissue injury [16,17].

Several studies with nematode-infected mice demonstrate the relevance of the balance between M1 and M2 macrophages and the molecules these cells release [18]. Notably, studies involving *Heligmosomoides polygyrus* [19] and *Nippostrongylus brasiliensis* [20], as well as the filarial nematodes *Brugia malayi* and *Litomosoides sigmodontis* [21], and the trematode *Schistosoma japonicum* [22] highlight an initial T-helper 1 (Th1)-M1 inflammation profile associated with tissue damage, followed by a Th2-M2 polarization that promotes tissue repair. It is clear that macrophages participate in many important immune functions following infection with helminths that are characterized by M2 signature molecules (e.g. including Arg1, FIZZ1, and Ym1) [23]. However, there are limited data regarding the implication of macrophage activation via STAT1 and STAT6 during the second larvae (L2) infective stage of *T. canis* infection. Previously, we reported that Th2 cytokines have a dual role during toxocariasis. On the one hand, STAT6 activation contributes to host susceptibility, and, alternatively, the down-regulated immune response reduces the immunopathology induced by *T. canis infection* in the lungs [24].

Despite these findings, many unresolved questions remain. It has not been determined whether M2 macrophages are the major cell-type involved in tissue repair through their activation via STAT6. Also, the role of M1 macrophages after L2 *T. canis* infection remains unknown. To better understand the possible roles played by M1 and M2 macrophages during acute toxocariasis, we used STAT1- or STAT6-deficient mice. Following L2 *T. canis* infection, STAT1 $^{-/-}$ mice favored M2 activation defined by increased lung expression of Arg1, FIZZ1, and Ym1, which resulted in parasite resistance and lung tissue repair. The STAT6 $^{-/-}$ mice displayed evidence of M1 activation and iNOS expression, which helped reduce the number of larvae in different tissues, but generated increased inflammation and extensive lung pathology. Therefore, M1 and M2 macrophages are involved in *T. canis*-associated

pathology. We suggest that during the lung migratory stage of *T. canis* a balance at a specific time between the inflammatory and anti-inflammatory response is necessary for protection against *T. canis*.

2. Results

2.1. Parasite Burden and Kinetics of Parasite Migration

To assess susceptibility (presence) or resistance (absence) to *T. canis* larvae, BALB/c WT, STAT1$^{-/-}$ and STAT6$^{-/-}$ mice were orally infected with 500 L2 larvae and the kinetics of parasite burden in the liver, lung, brain, and muscle was determined. Wild-type mice showed highest parasite burden in the liver and lung early in infection (4 dpi; Figure 1a,b) and diminishing thereafter. The brain and muscle were larger devoid of parasites at 4 dpi and increased gradually in these tissues over the next 56 days (Figure 1c,d). In contrast, although STAT1 $^{/}$ and STAT6 $^{/}$ mice displayed the same larval migration patterns as that of WT mice, the parasite burden in both strains was lower compared to WT mice. These data suggest that the absence of STAT1 and STAT6 did not affect the migration pattern of the larvae and that both STATs are crucial in eliminating the tissue stages of *T. canis*, making both STAT1 $^{/}$ and STAT6 $^{/}$ mice less susceptible to L2 *T. canis* infection.

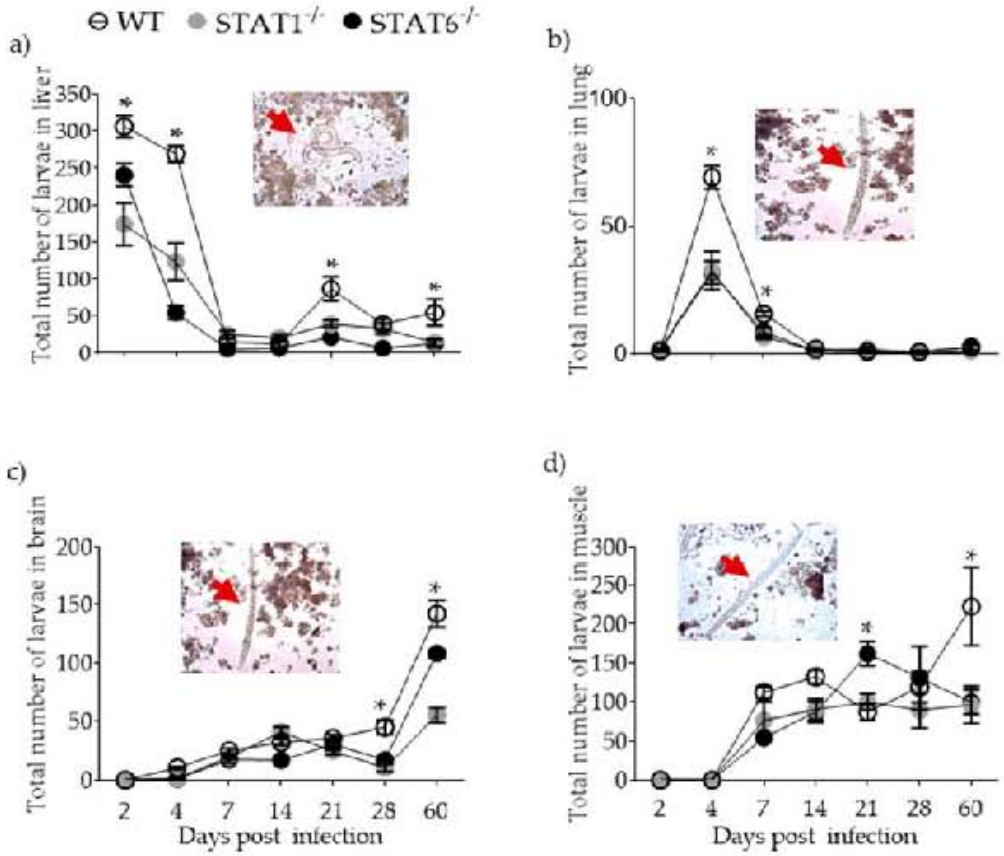

Figure 1. Kinetics of parasite migration. After infection with 500 L2 *T. canis* nematodes, the parasites were counted in the liver (a) lungs (b) brain (c) and muscle (d) in wild-type (WT) (solid white circles), STAT1$^{-/-}$ (STAT1 deficient mice, solid gray circles) and STAT6$^{-/-}$ (STAT6 deficient mice, solid black circles) mice at 2, 4, 7, 14, 21, 28, and 60 dpi. Red arrows indicate the presence of larvae in each tissue during the counting. Data are shown from two independent experiments as the mean ± SEM (n = 6 per group); two-way ANOVA with Tukey multicomparison test; * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice, at the same time point of infection.

2.2. Hemorrhagic Lesions and Inflammatory Infiltrates in Lung Tissue are Affected by STAT1 and STAT6

Having confirmed the migration of L2 *T. canis* through the lungs, lung pathology was assessed during acute infection. We observed the development of numerous hemorrhagic spots in the lung parenchyma of WT mice on 4 and 7 dpi, which gradually decreased over time (Figure 2a,b). Following infection with L2 *T. canis*, STAT1 $^{/}$ mice showed fewer and smaller lesions in the lungs on 4 and 7 dpi as compared to WT mice: lung lesions were not detectable on 28 dpi in STAT1$^{-/-}$ mice (Figure 2a,b). However, STAT6 $^{/}$ mice, despite having fewer larvae than WT mice (and similar to parasite burden of STAT1$^{-/-}$ mice), displayed numerous hemorrhagic lesions in the lungs that persisted until 60 dpi (end of experiment) (Figure 2a,b). Histological examination of the lungs revealed peribronchial and perivascular inflammation during acute infection (4–7 dpi) that healed as the infection progressed in both WT and STAT1$^{-/-}$ mice. Erythrocytes in the alveolar space and the presence of larvae surrounded by inflammatory infiltrate (Figure 2c–e) correlated with the hemorrhagic lesions observed in the macroscopic study. Interestingly, histological analysis of STAT6$^{-/-}$ mouse lungs revealed a marked inflammatory infiltrate that lasted for longer periods of time (4 to 60 dpi), suggesting impaired healing, despite parasite clearance. These findings suggest that the prompt or delayed healing of the lungs in STAT1 $^{/}$ or STAT-6 $^{/}$ mice, respectively, was associated with the type of immune response elicited, rather than the presence of L2 *T. canis* parasitic burden. Enumeration of lymphocytes (Figure S3a), macrophages (Figure S3b) and polymorphonuclear (PMN) (Figure S3c) cells revealed greater numbers of macrophages in lung tissue from day 4 to 21 post infection in WT, STAT1$^{-/-}$ and STAT6$^{-/-}$ mice.

2.3. Immune Response Associated with Lung Tissue Damage

To determine whether an inflammatory or anti-inflammatory immune response profile accompanied the delayed recovery of lung tissue in *T. canis*-infected STAT6$^{-/-}$ mice, cytokines and IgG isotypes of anti-*T. canis*-specific antibodies were measured in peripheral blood serum. Inflammatory cytokines such as IFN-γ (Figure 3a) and TNF-α (Figure 3b), as well IgG2a (Figure 3c), were increased in *T. canis*-infected STAT6$^{-/-}$ mice compared to WT and STAT1$^{-/-}$ mice (Figure 3a–c). The type 2 cytokines, IL-4 (Figure 3d), IL-10 (Figure 3e), and IL-13 (Figure 3f) and IgG1 (Figure 3g), were increased in WT and STAT1$^{-/-}$ mice but not STAT6$^{-/-}$ mice.

To assess the local immune response, cytokines were measured by RT-PCR in whole lung tissue. *T. canis*-infected STAT6$^{-/-}$ mice had increased expression of IFN-γ (Figure 4a) and TNF-α (Figure 4b) at the time when major macroscopic lesions occurred in the lungs. On the other hand, STAT1$^{-/-}$ mice displayed increased expression of IL-4 (Figure 4c) and IL-10 (Figure 4d,e), matching the reduced number of macroscopic lesions in the lungs. These results support the hypothesis that the delayed recovery in lung tissue from STAT6 $^{/}$ mice could be associated with a pro-inflammatory profile, while a prompt recovery in STAT1$^{-/-}$ mice may be associated with an anti-inflammatory profile during L2 infection with *T. canis*.

2.4. M1 and M2 Signature Activation Markers in Lung Tissue during Acute T. canis Infection

IFN-γ is critical for M1 macrophage activation and iNOS expression [7,9]. IL-4 and IL-13 are important for M2 macrophage differentiation and Arg1, FIZZ1, and Ym1 expression [17]. Thus, mRNA for these M1 and M2 markers were measured in lung samples by RT-PCR. iNOS mRNA expression was markedly increased in *T. canis*-infected STAT6 $^{/}$ mice (Figure 5a) corresponding temporally with the appearance of macroscopic lesions in the lungs. In contrast, the mRNAs for Arg1, FIZZ1 and Ym1 were increased in the lungs of WT and STAT1$^{-/-}$ mice (Figure 5b–e).

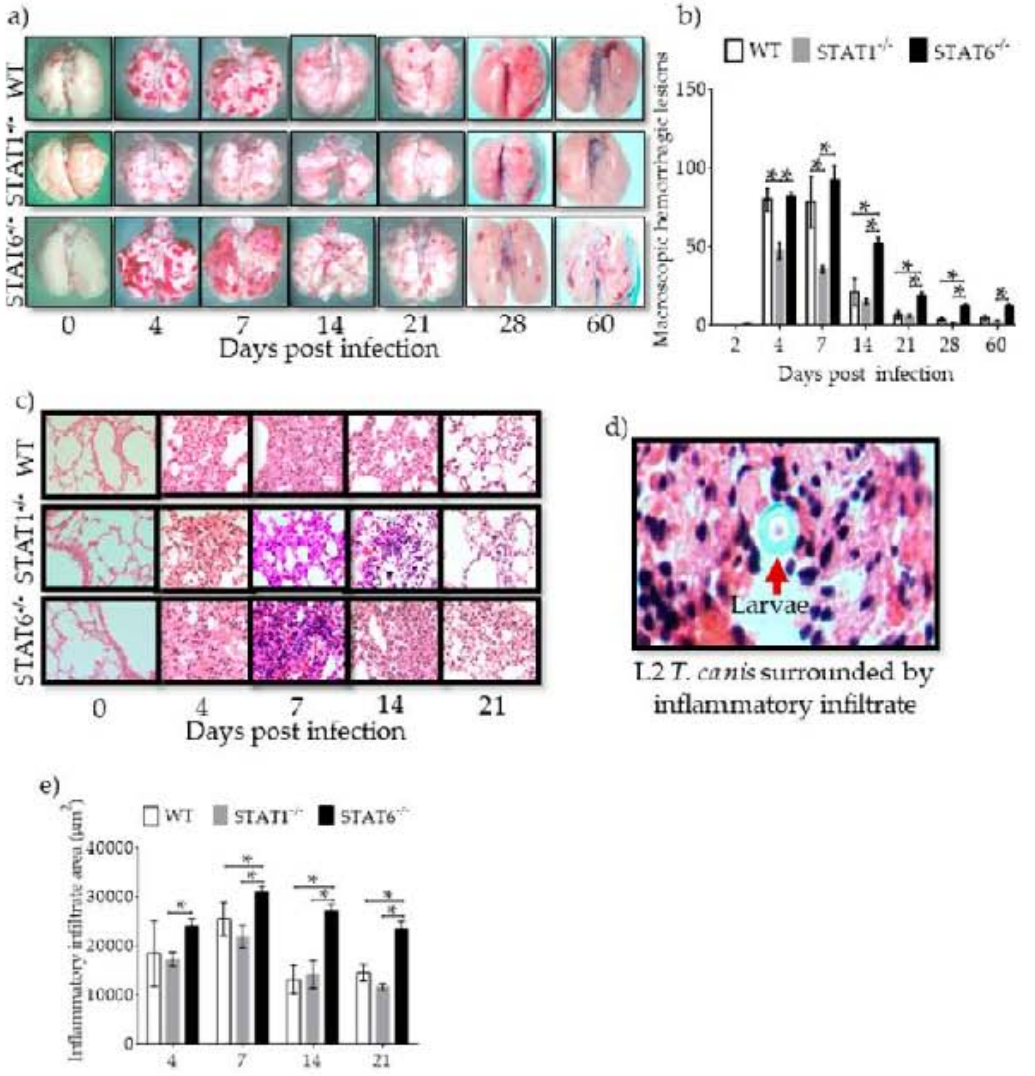

Figure 2. Hemorrhagic lesions and inflammatory infiltrates in lung tissue. Lungs were collected, washed with PBS and photographed at 0–60 dpi (**a**) and macroscopic hemorrhagic lesions were counted in WT (white bars), STAT1 $^{-/-}$ (gray bars) and STAT6 $^{-/-}$ (black bars) mice (**b**). Paraffin sections 4 µm thick from lung tissue were H&E stained, and inflammatory infiltration and hemorrhagic lesions were observed in microscopy light (40× objective) images at 0, 4, 7, 14, and 21 dpi in the three strains of mice (**c**). Representative image of the L2 *T. canis* surrounded by inflammatory infiltrate (60× objective) (**d**). Inflammatory infiltration was quantified from images taken at 40× objective in light microscope (**e**) in WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6-/- (black bars) mice. Data are shown from two independent experiments as the mean + SEM (n = 6 per group). Two-way ANOVA with Tukey's multicomparison test. * $p < 0.05$ comparing WT versus STAT1 $^{-/-}$ and STAT6 $^{-/-}$, and STAT1 $^{-/-}$ versus STAT6 $^{-/-}$ mice at the same time point of infection.

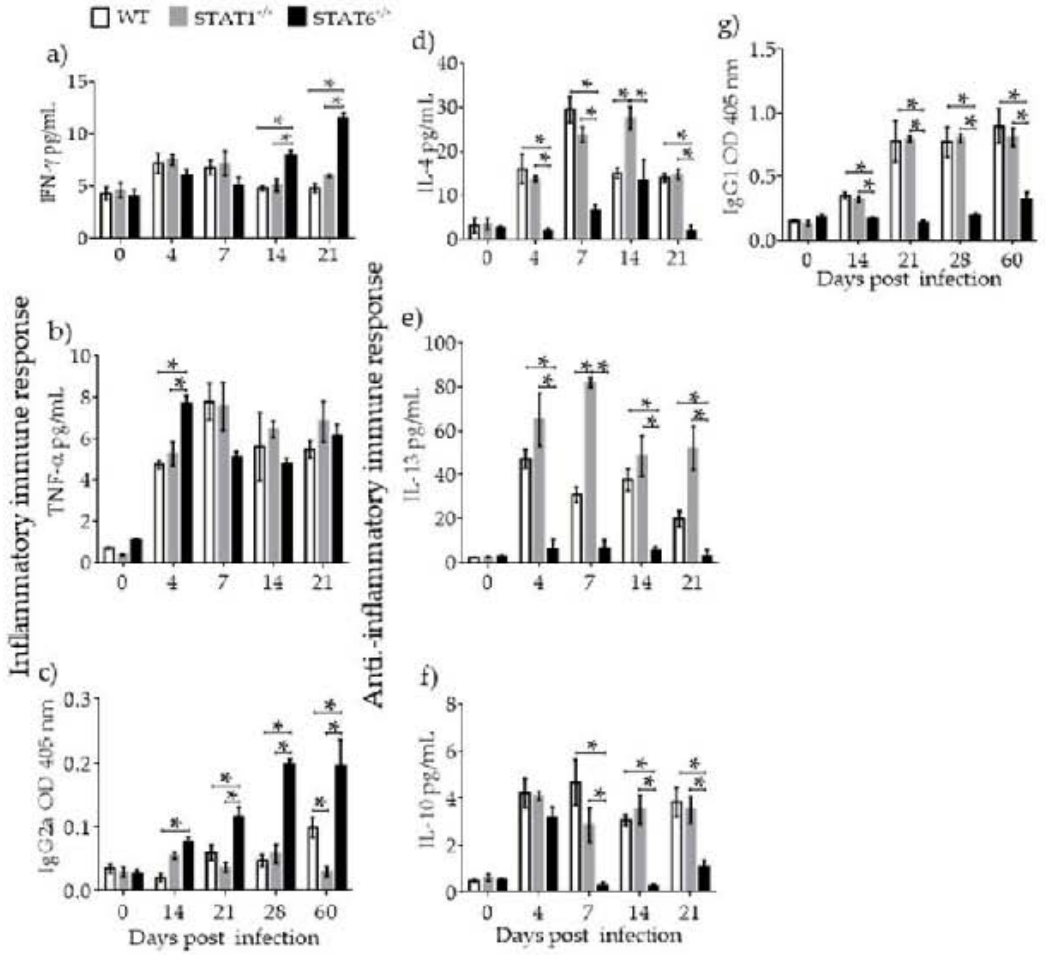

Figure 3. Immune response associated with lung tissue damage during *T. canis* infection. Cytokines and antibodies of inflammatory (interferon (IFN)-γ (a), TNF-α (b), IgG2a (c)) and anti-inflammatory IL-4 (d), IL-13 (e), IL-10 (f), IgG1 (g) immune responses were measured in serum at 0–60 dpi from WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice. Data are shown from two independent experiments as the mean ± SEM. For cytokines: (n = 4–6 per group); unpaired t-test with Holm–Sidak multicomparison test. For antibodies: (n = 6 per group); two-way ANOVA with Tukey's multicomparison test. * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice at the same time point of infection.

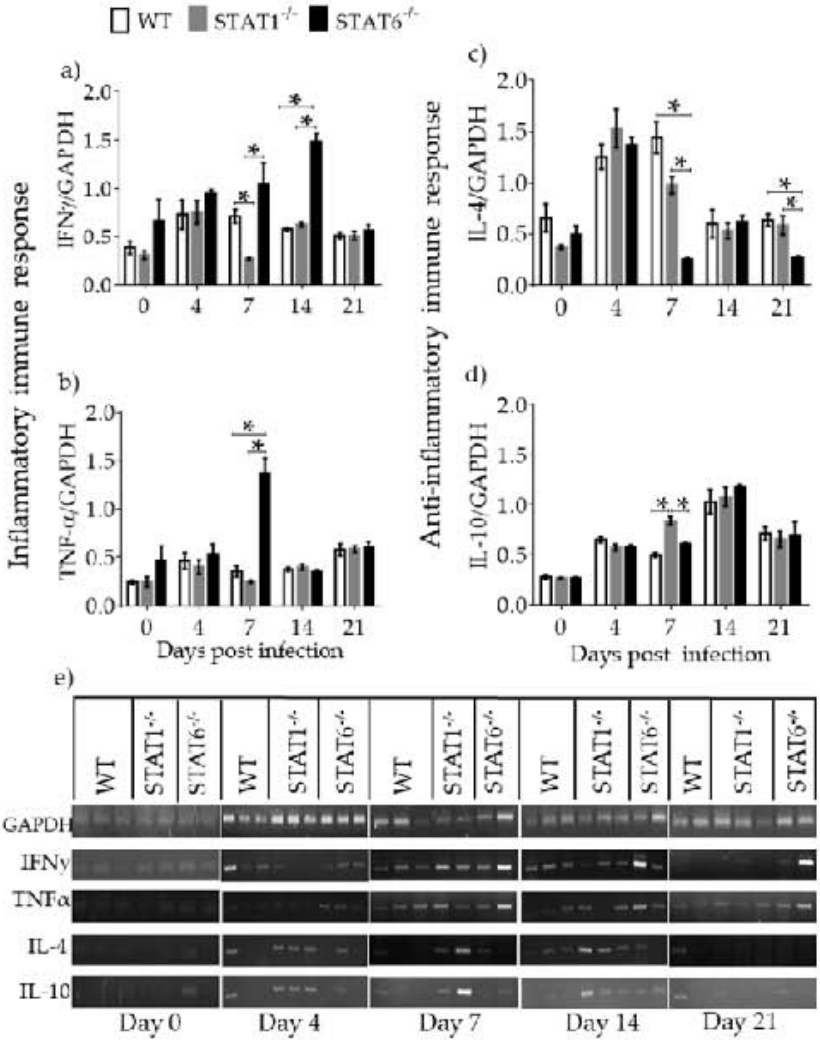

Figure 4. Local immune response associated with lung tissue damage. mRNA expression of IFN-γ (a), TNF-α (b), IL-4 (c) and IL-10 (d) was evaluated in whole lung tissue of WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice at 0, 4, 7, 14, and 21 dpi. A representative electrophoresis gel of the various genes and GAPDH control from one representative experiment is shown (e) Data are shown from two independent experiments as the mean ± SEM (n = 5–6 per group); unpaired t-test with Holm–Sidak multicomparison test.; * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice at the same time point of infection.

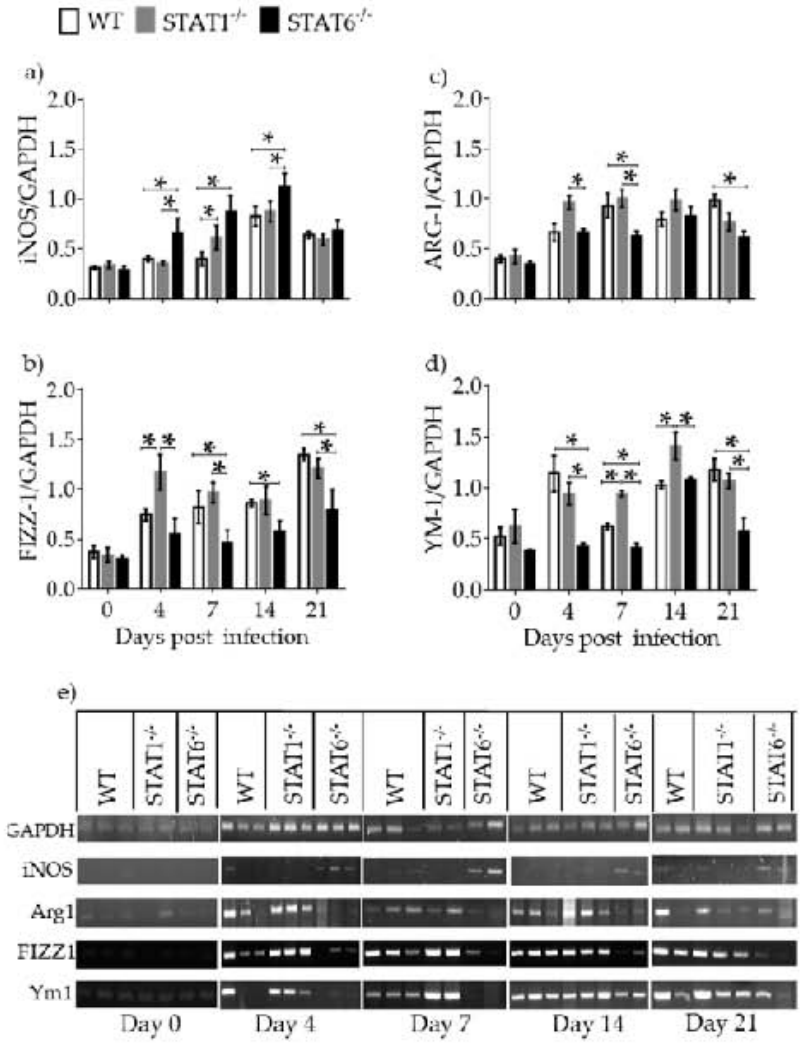

Figure 5. Classically activated (M1) and alternatively activated (M2) macrophage activation markers in lung tissue. mRNA expression of inducible nitric oxide synthase (iNOS) (a) Arg1 (b) FIZZ1 (c) and Ym1 (d) was evaluated by RT-PCR in whole lung tissue from WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice at day 0, 4, 7, 14, and 21 dpi. A representative electrophoresis gel of the different previously mentioned genes and their respective GAPDH control from one experiment is shown (e) Data from graphs are shown from two independent experiments as the mean ± SEM (n = 5–6 per group); unpaired t-test with Holm–Sidak multicomparison test.; * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$, versus STAT6-/- mice at the same time point of infection.

Lung sections were subsequently assessed by immunofluorescence. iNOS protein expression was strongly detected by 4 dpi, and remained elevated in the lungs it was sustained at least until 14 dpi in STAT6$^{-/-}$ mice (Figure 6a,b). In contrast, STAT1$^{-/-}$ mice had reduced expression of iNOS, while WT mice displayed discrete and transient iNOS expression, particularly at 4 dpi (Figure 6a,b).

Regarding Ym1 expression, which has been consistently associated with tissue repair [25], we found that its early expression in the lungs was largely dependent on STAT6 signaling, given that Ym1 expression was significantly reduced in the lungs of STAT6 $^{-/-}$ mice. Ym1^{+} cells were abundant in both WT and STAT1$^{-/-}$ mice, especially at 4 and 7 dpi (Figure 7a,b).

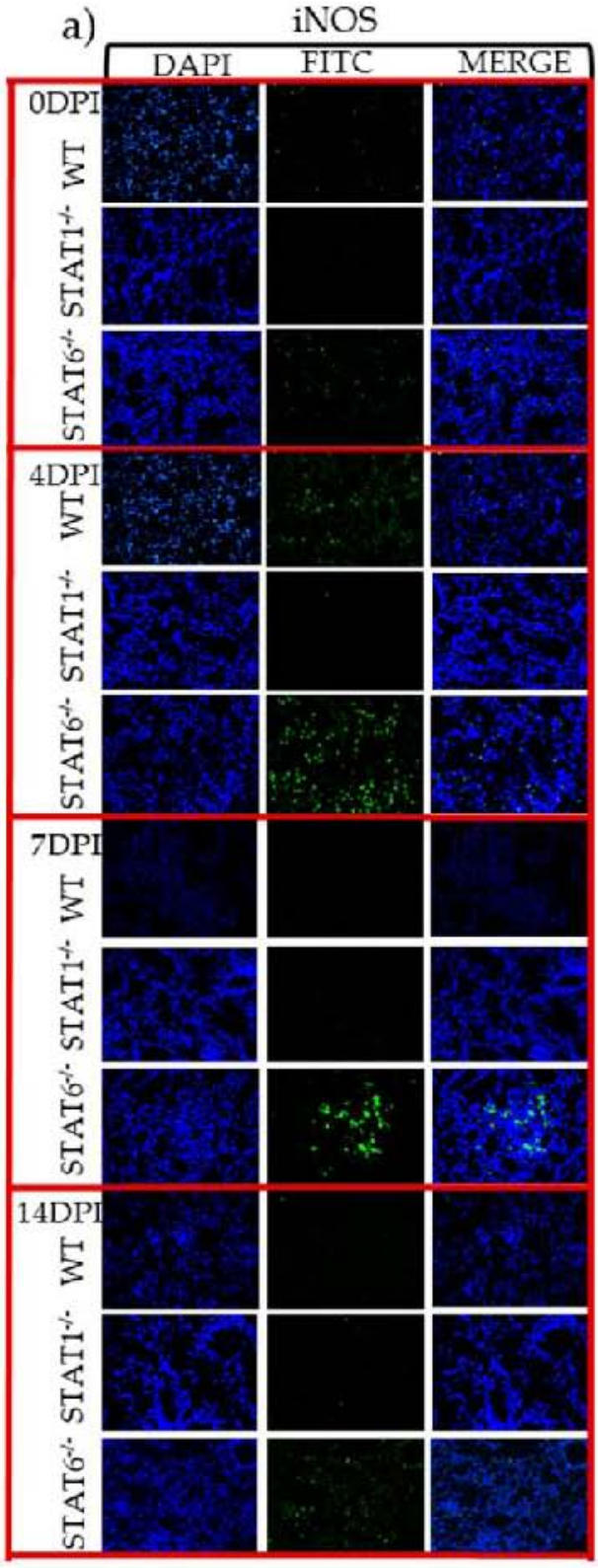

Figure 6. *Cont.*

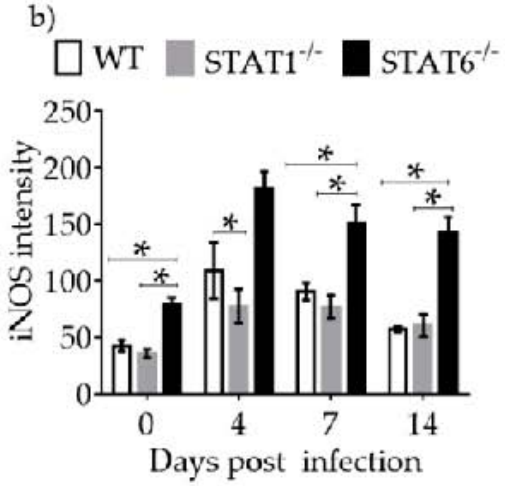

Figure 6. M1 macrophage activation marker in lung tissue assessed by immunofluorescence. Microscopy data of lung sections from WT, STAT1$^{-/-}$ and STAT6$^{-/-}$ mice at 4, 7 and 14 dpi, stained with the DNA-binding dye (DAPI) in blue and iNOS (a) in green. Quantification of the fluorescent intensity of iNOS (b) in lung sections stained from a, in WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice. Photographs were taken with a 20× objective. Data are shown from two independent experiments as the mean ± SEM (n = 6 per group). Two-way ANOVA with Tukey's multicomparison test. * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice at the same time point of infection.

To confirm the presence of macrophages and to assign them a probable role in either lung tissue damage or repair, F4/80-CD86 double-positive cells for M1 activation and F4/80-IL4Rα and F4/80-MMR cells double-positive for M2 activation were evaluated by flow cytometry (Figure 8a–e). STAT6$^{-/-}$ mice displayed an increased percentage of F4/80^{+}CD86^{+} cells that was not observed in either WT or STAT1$^{-/-}$ mice (Figure 8b) at 7 dpi. However, WT and STAT1$^{-/-}$ mice showed an increased percentage of both F4/80^{+}IL4Rα^{+} and F4/80^{+}MMR^{+} cells (Figure 8c–d) at the same time point after *T. canis* infection.

Collectively, these data suggest that STAT6 deficiency promotes M1 macrophage activation and lung tissue damage, while the absence of STAT1 is associated with M2 macrophage polarization and early lung tissue repair during acute toxocariasis.

2.5. Differential Expression of Molecules Associated with Tissue Repair and Fibrosis

Asking if the repair mechanisms contributed to fibrosis generation, factors associated with fibrosis development and tissue repair, namely collagen, elastin, MMP-9, and TGF-β, were assessed. Elastin, a wound repair factor, increased in all strains of mice at 21 dpi. *T. canis*-infected STAT6$^{-/-}$ mice had a smaller increase in elastin compared with WT and STAT1$^{-/-}$ mice at 4 dpi (Figure 9a,c). Masson's trichrome staining did not reveal differences in collagen deposition between the strains (Supplementary Figure S1). MMP9, an enzyme that degrades elastin, reached peak of expression at 14 dpi in all strains of mice. However, STAT1$^{-/-}$ and STAT6$^{-/-}$ mice displayed higher levels of MMP9 at 4 and 7 dpi (Figure 9b,d). Whereas TGF-β mRNA a factor associated with tissue repair produced by M2 macrophages [18] was modestly expressed in WT mice, its expression was significantly enhanced in STAT1$^{-/-}$ mice at different dpi. Interestingly, STAT6$^{-/-}$ mice failed to enhance TGF-β expression in lung tissue (Figure 9e,f). These observations suggest that neither experimental group of mice promotes greater fibrosis.

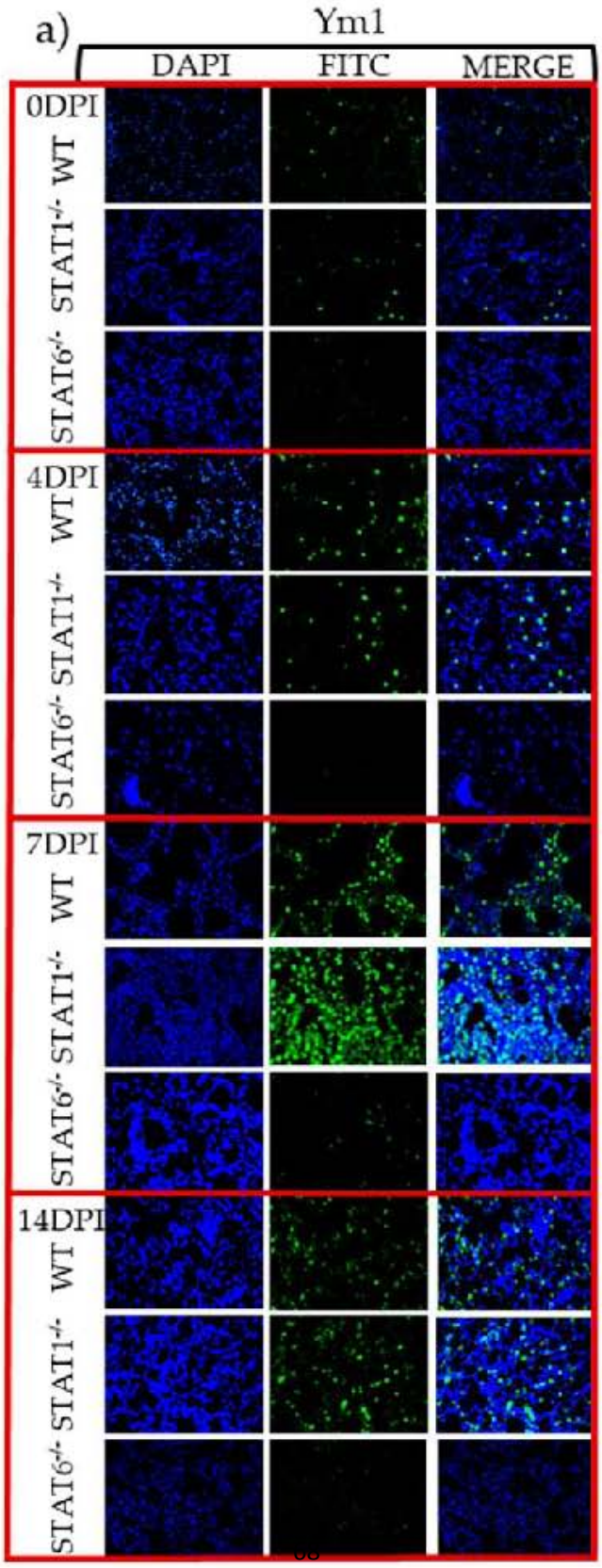

Figure 7. *Cont.*

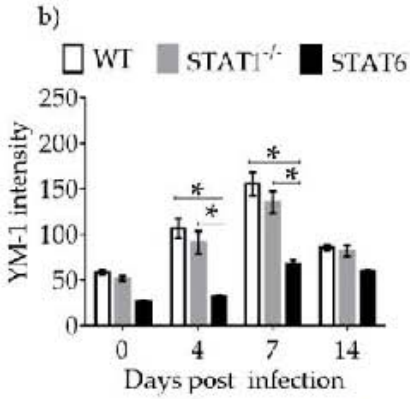

Figure 7. M2 macrophage activation marker in lung tissue assessed by immunofluorescence. Microscopy data of lung sections from WT, STAT1$^{-/-}$, and STAT6$^{-/-}$ mice at 4, 7, and 14 dpi, stained with the DNA-binding dye (DAPI) in blue and Ym1 (a) in green. Quantification of the fluorescent intensity of Ym1 (b) in lung sections stained from a, in WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice. Photographs were taken with a 20× objective. Data are shown from two independent experiments as the mean ± SEM (n = 6 per group). Two-way ANOVA with Tukey's multicomparison test. * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice at the same time point of infection.

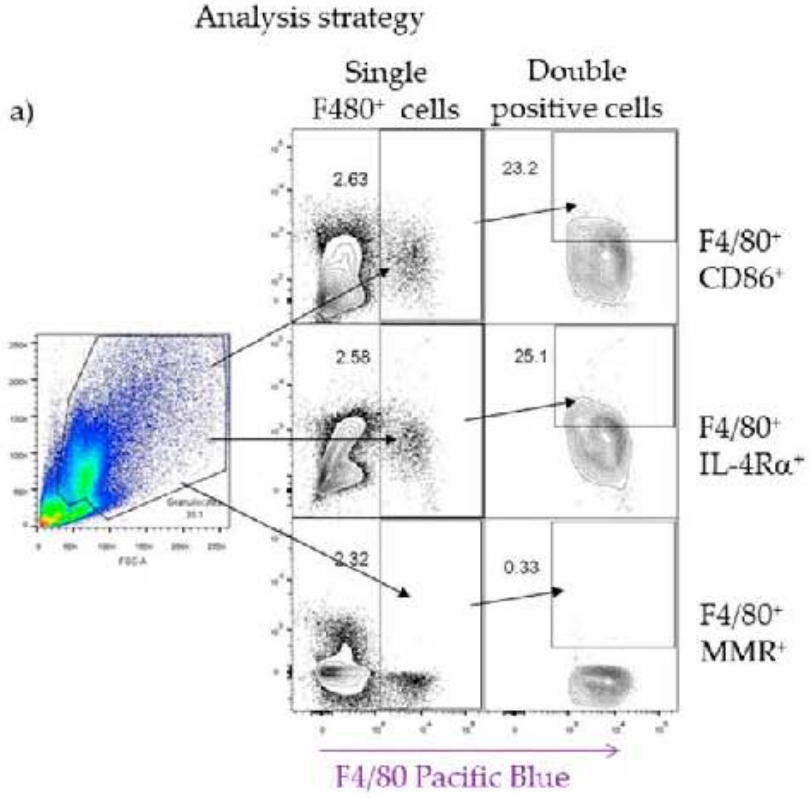

Figure 8. *Cont.*

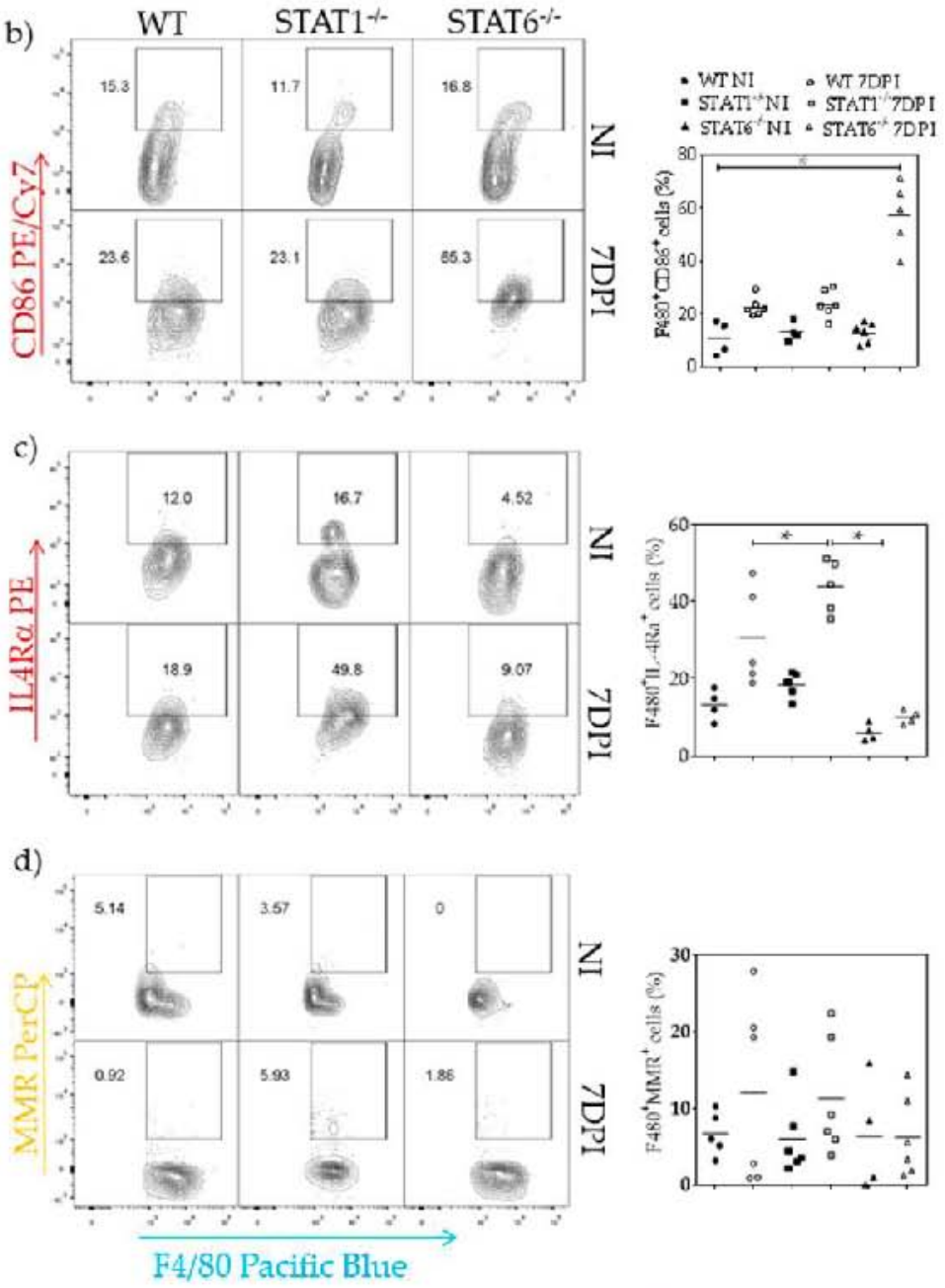

Figure 8. Detection of cell surface markers associated with M1 and M2 macrophage activation. Mice were infected with 500 L2 *T. canis* larvae, and lung cells were obtained at 7 dpi for flow cytometry staining. Representative FACS analysis of the cell surface markers F4/80, CD86, IL-4Rα, and MMR. The macrophage region was first defined by FSC and SSC characteristics and further subgated based on the F4/80 cell population. Then, one thousand events from either subgate were captured (a) Representative dot plots and their respective percentage of $F4/80^{+}CD86^{+}$ (b), $F4/80^{+}IL\text{-}4R\alpha^{+}$ (c), and $F4/80^{+}MMR^{+}$ (d) double-positive cells in infected and noninfected (NI) mice. Each dot plot represents an individual mouse. Data are shown from two independent experiments of 5–6 animals per group. One-way ANOVA and Tukey's multiple comparison test. * $p < 0.05$ comparing WT versus STAT1 $^{-/-}$ and STAT6 $^{-/-}$ mice.

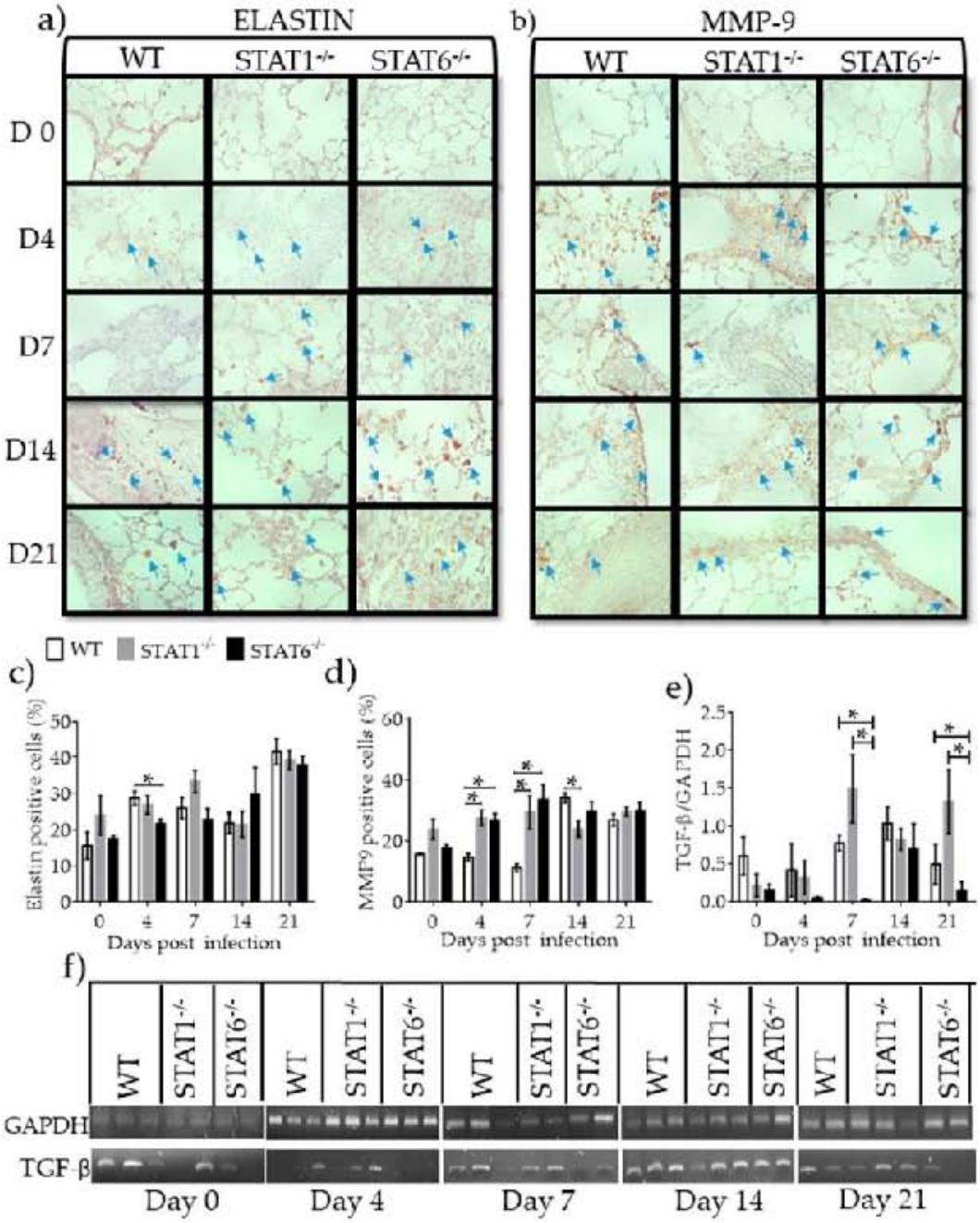

Figure 9. Molecules associated with tissue repair and fibrosis. Paraffin sections of lung tissue were processed to measure elastin (left panel) and MMP9 (right panel) by IC as markers of fibrosis. Representative images from lung tissue sections were photographed with the 60× objective (a,b) and the percentage of Elastin- and MMP9-positive cells (brown marks pointed by arrows) was measured at different dpi in WT (white bars), STAT1$^{-/-}$ (gray bars) and STAT6$^{-/-}$ (black bars) mice (c,d) mRNA expression of TGF-β as a marker of tissue repair was measured by RT-PCR (e) and its respective electrophoresis gel is shown (f) Data are from two independent experiments presented as the mean ± SEM (n = 5–6 per group); unpaired t-test with Holm–Sidak multicomparison test; * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$, and STAT1$^{-/-}$ versus STAT6$^{-/-}$ mice at the same time point of infection.

2.6. Lung Macrophage Depletion Alters the Inflammatory and Repair Process in Acute Toxocariasis

To examine whether macrophages were involved in a rapid lung tissue repair process during acute L2 *T. canis* infection, macrophages were depleted by intratracheal administration of clodronate-loaded liposomes. Infected WT and STAT1$^{-/-}$ mice treated with PBS-liposomes displayed well-defined hemorrhagic lesions, while those receiving the clodronate-liposomes displayed severe, large lesions that were more diffuse and widespread throughout the lungs (Figure 10a). In *T. canis*-infected STAT6$^{-/-}$ mice treated with PBS-liposomes, the hemorrhagic lesions were obvious, numerous and widely distributed throughout the lung, compared to WT and STAT1$^{-/-}$ mice (Figure 10a). Conversely, infected STAT6$^{-/-}$ mice treated with clodronate-liposomes developed fewer hemorrhagic lesions compared with those of *T. canis*-infected STAT6$^{-/-}$ and WT mice receiving PBS-loaded liposomes. Notably, administration of clodronate-loaded liposomes in STAT6$^{-/-}$ mice reduced hemorrhagic lesions, and the appearance of the lungs was similar to that of *T. canis*-infected WT and STAT1$^{-/-}$ mice (Figure 10a), suggesting that macrophages may mediate control of hemorrhaging and the inflammation caused by the lung stage of this helminth in a STAT6-dependent manner. In accordance with these findings, inflammatory infiltrates were clearly affected by macrophage depletion. Thus, *T. canis*-infected WT mice treated with clodronate liposomes displayed increased inflammatory infiltrates compared to those of *T. canis*-infected WT mice receiving PBS-liposomes. Similarly, the elimination of macrophages in *T. canis*-infected STAT1$^{-/-}$ mice resulted in an increased inflammatory infiltrate in the lungs. In contrast, *T. canis*-infected STAT6$^{-/-}$ mice treated with clodronate liposomes displayed a marked reduction in inflammatory cell infiltration (Figure 10b,e). Flow cytometry analysis was performed to assess macrophage depletion. Mice receiving clodronate-liposomes had a significant reduction in the F4/80^{+} cell population in all three infected strains of mice, ranging from 50%–74% reduction in lung macrophage numbers compared to that of their infected PBS-liposome control groups (Figure 10c,d). Consequently, analysis of F4/80^{+}CD86^{+}, F4/80^{+}IL4-Rα^{-}, and F4/80^{+}MMR^{+} double-positive cells revealed no significant differences in either the clodronate liposome-treated groups or between strains (Supplementary, Figure S2).

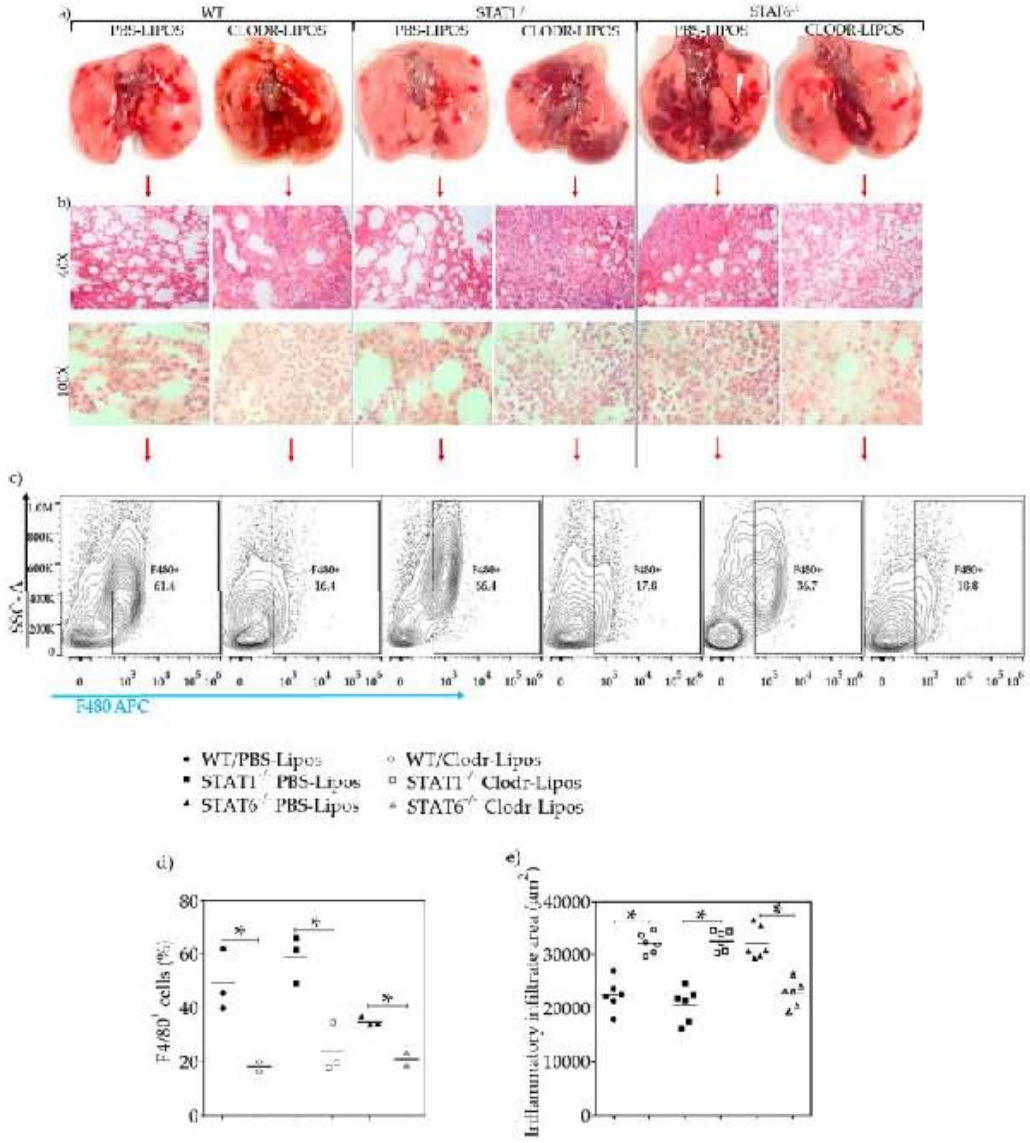

Figure 10. Lung macrophage depletion. WT, STAT1$^{-/-}$, and STAT6$^{-/-}$ mice were treated either with PBS-liposomes or clodronate-liposomes intratracheally to deplete macrophages, before and after being infected with 500 L2 *T. canis* larvae. The animals were euthanized at 4 dpi, and the lungs were taken and photographed, (a) H&E staining was carried out to obtain images at 40× or 100× (b), and the inflammatory infiltrate areas were measured (e). Lungs cells were collected, and flow cytometry was used to determine the efficacy of the treatment. Representative dot plots and their respective percentage of F4/80-positive cells of the three strains of mice (c,d) * $p < 0.05$ comparing WT versus STAT1$^{-/-}$ and STAT6$^{-/-}$ mice.

3. Discussion

Macrophage phenotype is dictated by the conditions of the micro-environment and the local cytokine milieu [26]. The M1 macrophage has robust anti-microbial and anti-tumor activity, can cause ROS-induced tissue damage, and impair tissue regeneration and wound healing [27,28]. By contrast, the regulatory anti-inflammatory M2 macrophage promotes tissue restitution after damage [28–31].

Some authors divided M2 macrophages into wound healing macrophages (M2a) and regulatory macrophages (M2b) [7,32]; others describe macrophage polarization as a "spectrum model" with at least nine distinct macrophage activation programs in humans [33]; Reyes et al [34], Martinez et al,

and Mantovani et al [35] reported that M2a macrophages reflects the typical alternative profile induced by IL-4 and IL-13, M2b induced by exposure to immune complexes release high levels of IL-10 and M2c represents the original version of deactivated macrophages induced by IL-10 and glucocorticoid hormones [7,34,35]; Murray et al propose that the term regulatory macrophages should be avoided because all macrophage populations have regulatory capacities and a correct use of the nomenclature concerning M1/M2 is needed to avoid confusions [36]. Following the previous studies and the proper suggestions, for the purpose of the present study we decided to study the common M1/M2 classification, activated by IFN-γ and IL-4 or IL-13, respectively.

Helminth migration through the host on route to its preferred niche can cause significant tissue damage and so it is imperative that the host be capable of initiating rapid and effective tissue repair. Analyses of helminth-rodent model systems has yielded considerable knowledge of the cellular and molecular players responsible for tissue recovery after injury [37,38]. Not surprisingly the mobilization of repair mechanisms is host-parasite specific [39], and several distinct pathways of tissue repair have been described. Yet, there is a paucity of studies of assessing tissue recovery in response to acute toxocariasis [5,6,40].

In the present study, the role of M1 and M2 macrophages in the repair process during the lung stage of *T. canis* infection was assessed using STAT1$^{-/-}$ and STAT6$^{-/-}$ mice. According to the findings, the M1 macrophage population induces damage in the lungs during the early stages of L2 *T. canis* migration. In STAT6$^{-/-}$ mice, even when the larvae were almost absent from the lungs in the chronic stages, tissue damage persisted and correlated with increased iNOS indicative of M1-type cells. Consistent with these observations, macrophage depletion in *T. canis*-infected STAT6$^{-/-}$ mice resulted in a reduction in the number of hemorrhagic lesions in the lungs and better healing. Together, these data strongly suggest that M1 macrophages are a major contributor to lung damage during the early and chronic stages of L2 *T. canis* infection. Despite the damage produced in the absence of STAT6, the mechanisms of tissue repair associated with an anti-inflammatory immune response were not completely suppressed. Thus, markers associated with wound healing such as Arg1, FIZZ1, and Ym1 were detectable, as was TGF- mRNA, and the regenerative process associated with MMP9 and elastin expression were not altered, resulting in delayed, not absent, healing processes in the infected STAT6$^{-/-}$ mice. These data are supported by a recent report from Sutherland et al., showing that Ym1 and FIZZ1 expressions are not completely dependent on the IL4R-α signaling pathway [41].

Other signaling pathways, in addition to STAT6, are implicated in M2 macrophage activation and the repair mechanisms needed when tissues are damaged, that involve, for example, Akt [42], STAT3 [43], IRF, and PPARγ [8] signaling. In additional, epithelial alarmins are secreted following infection with parasitic nematodes to activate type 2 innate lymphoid cells (ILC2) and drive type 2 immunity [37,44,45], which via IL-5 and IL-13 can mobilize wound healing mechanisms with the capacity to produce Arg1 [44] and FIZZ1 [46] to maintain tissue integrity. Consequently, in STAT6$^{-/-}$ mice treated with clodronate-liposomes, depletion of macrophages mainly reduces the population causing inflammation, promoting a more efficient activation of all repair mechanisms and resulting in a reduction in hemorrhagic lesions. Similar results were reported in *N. brasiliensis*-infected STAT6$^{-/-}$ mice where a predominant inflammatory profile was observed coupled to persistence of pathology [47]. Thus, increased inflammation drives incomplete M2 macrophage activation.

In our model of infection with *T. canis*, we evaluated the role of M2 macrophages in lung tissue repair by suppressing the inflammatory response through STAT1$^{-/-}$ mice. We observed that the regenerative process in this strain of mouse was faster compared with STAT6$^{-/-}$ and WT mice. In STAT1$^{-/-}$ mice, an anti-inflammatory profile was apparent; IL-13 expression was increased, Arg1, FIZZ1 and Ym1 were highly expressed (or showed a tendency to be increased), and F4/80^{+}IL-4Rα^{+} cells were highly increased and F4/80^{+}CD86^{+} cells decreased. Finally, when macrophages were depleted with clodronate-liposomes, major lung pathology manifested. These data from STAT1$^{-/-}$ mice confirm that M2 macrophages play a crucial role in lung tissue healing during *T. canis* infection, and their absence compromises the process of tissue regeneration.

The anti-inflammatory immune response, commonly observed following infection with parasitic helminths, regulates tissue repair via the coordinated activity of Th2 cells, eosinophils, mast cells, basophils, ILCs2, and M2 macrophages [48,49]. Nevertheless, when type 2 cytokine-mediated repair processes become chronic, over exuberant or dysregulated, they can contribute to the development of fibrosis [49]. Diverse mechanisms inducing inflammation and fibrosis resolution are dependent on the etiological agent involved, tissue tropism, and the type of immune response triggered in each case [50]. Other factors associated with tissue repair, such as MMP2 and MMP9, can be produced by M2 macrophages, are involved in keratinocyte migration and contribute to the elimination of nonviable tissue [51–53]. In normal conditions, elastin is an essential protein of connective tissue that provides elasticity and support to diverse organs, including the lungs. During pulmonary pathologies, an increase in elastin deposition can cause fibrosis, and to avoid this, MMP9 and MMP2 are activated for protein degradation and restructuring of the extracellular matrix (ECM) [54]. Here, we observed that MMP9 and elastin are produced in similar quantities in all three strains of mice during the early phases of *T. canis*-infection. Thus, it is feasible that such a phenomenon might be a consequence of the damage induced by the helminth, which in turn promotes the activation of mechanisms that are necessary for tissue repair. We speculate that the increase in MMP9 counteracted the effect of elastin and collagen deposition, to avoid an obvious increase in fibrosis. Whereas TGF-β that was mainly expressed in WT and STAT1$^{-/-}$ mice may participate as a regulatory mechanism to facilitate the resolution of pro-inflammatory response and driver of tissue repair [31], therefore, the absence of STAT6 compromise its expression and tissue repair may be by M2 macrophages.

Another novel finding was that the immune response in STAT6$^{-/-}$ mice drove the clearance of the *T. canis* larvae. Previously, we reported corroborating data on parasite burden and concluded that IgE production was not essential for parasite neutralization because STAT6$^{-/-}$ mice did not produce IgE [24]. A possible mechanism underlying the resistance to this parasitic infection in STAT6$^{-/-}$ mice is increased activation of M1 macrophages, which favors iNOS expression. Few studies have proposed a role for NO in the resistance against nematode parasites. In an in vitro study, Pfaff et al. showed that *L. sigmodontis* microfilariae were vulnerable to NO. However, neither pharmaceutical inhibition of nitric oxide synthesis nor iNOS knockout in mice abrogated resistance to circulating *L. sigmodontis* [55]. More recently, iNOS$^{-/-}$ mice infected with *Strongyloides venezuelensis* showed increased susceptibility to infection [56]. These results illustrate that mechanisms of protection or susceptibility defined in one model system cannot be simply assigned to all helminth-host interactions: rather particular differences can be critical in favoring initial inflammatory responses that have a major role in inducing protection during helminth infections. The data herein, can be interpreted in favor of iNOS contributing to the resistance of STAT6$^{-/-}$ mice to infection with L2 *T. canis*.

Moreover, we observed that STAT1$^{-/-}$ mice had a decreased parasite burden, similar to that of STAT6$^{-/-}$ mice. These results can be explained by an exacerbated anti-inflammatory immune response that is the archetypical response against helminth parasites. For instance, FIZZ1 is a reputedly important molecule in eliminating *S. mansoni* or *N. brasiliensis* from the intestine of mice and this effect was linked to an enhanced Th2 immune response [57]. In fact, in the absence of STAT1 we found an acutely increased expression of FIZZ-1, thus it may be that recently hatched larvae are rapidly expelled from the intestine, and therefore lungs, due to an increase in mucus production and fewer larvae may reach blood vessels for dissemination to other organs. In line with this Filbey et al. found that migrating *N. brasiliensis* larvae were killed in the lungs of mice co-infected with *H. polygyrus*, and the lung pathology associated with *N. brasiliensis* larval migration was reduced by robust Th2 immunity [58]. Finally, Esser-von Bieren et al suggested a novel IL-4Rα-independent mechanism of M2 activation that is antibody-dependent and mediates both anti-helminth immunity and tissue disruption caused by migrating *H. polygyrus* larvae [59].

Traditionally eosinophils are cited as an important anti-helminth effector mechanism [60–62] but more recent studies suggest the situation is not so simple; while infection with *T. canis* is associated with an eosinophilic response, these cells may have a limited capacity to kill the helminth and the

larvae can efficiently escape eosinophils in vitro [63–65]. In a recent and interesting review, it has been proposed a combined role for macrophages and eosinophils as key players in the mechanisms of granuloma formation during helminth infections and in the balance between parasite killing and healing [66]. Collectively, a scenario is forming in which inflammatory and anti-inflammatory immune responses are important in mediating tissue and helminth destruction. Thus, during *T. canis* infection, M2 macrophages activated via STAT6 are important cells in modulating lung tissue damage and conferring resistance to infection by inducing a potent anti-inflammatory immune response, whereas M1 macrophages activated via STAT1 promote lung pathology, while at the same time being important for parasite eradication. However, a complementary and more extended study, reviewing the role of other cell populations in conjunction with macrophages that can help to mediate parasite killing and wound healing during L2 *T. canis* infection like eosinophils or ILC2 cells, is needed.

In summary, our work extends awareness of the role(s) of the STAT1 and STAT6 pathways in lung healing during acute *T. canis* infection and highlights the importance of M2 macrophages in accelerating tissue recovery during the lung stage of this infection.

4. Materials and Methods

4.1. Animals

Eight-to-ten-week-old wild-type (WT), STAT1$^{-/-}$, and STAT-6$^{-/-}$ male BALB/c mice were purchased from the Jackson Laboratory Animal Resources Center (Bar Harbor, ME, USA) and maintained in a specific pathogen-free environment at the FES-Iztacala, U.N.A.M animal facility according to Faculty Animal Care and Use Committee and government guidelines (official Mexican regulation NOM-062-ZOO-1999) in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA).

4.2. Egg Isolation and Parasite Infection

Adult *T. canis* female worms were isolated from the intestines of naturally infected puppies (≤3 months old). Female worms were dissected to isolate immature eggs from the uterus and kept in distilled water. The mixture was washed and centrifuged twice at 1500 rpm for 10 min in 1% NaCl solution. After removal of the supernatant, the sediment was washed twice with distilled water and placed in a 1% formalin solution according to previous protocols [67] in a tissue flask at 28 °C for one month, with gentle daily agitation until L2 development inside the eggs, which was monitored under the microscope. After maturation, the mice were infected with 500 larvated eggs, administered intragastrically with a Foley tube.

4.3. Kinetics of Parasite Migration

Animals were euthanized at 0 (uninfected), 2, 4, 7, 14, 21, 28 and 60 days post infection (dpi). Lungs, liver, muscle and brain were cut into small pieces and placed in tubes containing 1% chloride acid (J.T. Baker; Edo. De Mex, Mexico) and 3% pepsin (Sigma Aldrich; St Louise; USA). To facilitate the release of L2 parasites from the different organs, tissues were incubated for 48 h at room temperature with agitation. After incubation, the samples were centrifuged at 1500 rpm for 10 min, the sediment recovered, reconstituted, and fixed with 10% formalin (Sigma Aldrich; Mexico City, Mexico), and the L2 parasites counted under a 10× objective in an optical microscope (Motic B5 Professional Series).

4.4. Macroscopic Lung Study

Complete lungs were taken from the mice, washed with ice-cold saline solution, photographed under a 4x objective using a stereoscopic microscope (ZEIGEN), and hemorrhagic lesions on the surface of the lungs were counted.

4.5. Histology, Hematoxylin and Eosin (H&E), and Masson's Trichrome Staining

The lungs were perfused via trachea and fixed with absolute alcohol, followed by paraffin embedding and 4 µm-thick sections were taken through the hilum and stained with the H&E for conventional histology analysis or Masson's trichrome stain to observe collagen deposition. Morphological features were assessed using an optical microscopic (LAS V4.9, Leica), and morphometric analysis was performed with the Leica program to determine the total surface and calculate the area of inflammatory infiltrate per μm^2. Cell counting of PMN lymphocytes and macrophages were carried out in a blinded fashion, at the light of microscope at 100× objective to determine the most common cellular phenotypes activated during the infection. PMN were identified by the shape of their nucleus bi-, tri-, or multi-lobulated, whereas macrophages cells were identified by size and were hemosiderin-laden macrophages.

4.6. Immunohistochemistry (IC) and Immunofluorescence (IF)

Four-micron thick lung sections were, deparaffinized and incubated with 10x DIVA Decloaker (Biocare Medical; CA, USA) at a 1:10 dilution. For IC, the slides were washed with PBS (3 × 5 min) peroxidase inhibition was performed with 10% hydrogen peroxide for 30 min, the slides washed with PBS (3 × 5 min) and blocked with PBS 3% BSA for 1 h at room temperature. The slides were incubated with purified primary rabbit anti-Elastin at a 1:200 dilution (Abcam; MA, USA) or goat anti-MMP9 at a 1:100 dilution (Santa Cruz; CA, USA) at 4 °C overnight. Next, the slides were washed with PBS (3 × 5 min) and secondary mouse anti-rabbit at a 1:1500 dilution (Biolegend; CA, USA) or mouse anti-goat at a 1:1500 dilution (Biolegend; CA, USA) were added and incubated for 1 h at room temperature. The slides were washed and incubated for 5 min with 50 µL of diaminobenzidine (DAB) chromogen kit (Biocare Medical; CA, USA) according to the manufacturer's instructions. Finally, counterstaining with Harris hematoxylin was performed. Photographs were obtained with an optic microscope (Axio Vert. A1, Carl Zeiss) using a 40× objective and analyzed with the program ImageJ. For IF, the slides were washed with PBS (3 × 5 min) and membrane permeabilization performed with PBS containing 2% Triton (Reasol; Mexico City, Mexico). Sections were washed with PBS (3 × 5 min) and blocked with PBS containing 3% BSA for 1 hour at room temperature. Different tissue sections were incubated with purified primary antibodies labeled with FITC fluorochrome rabbit anti-iNOS at a 1:100 dilution (Cell Signaling; MA, USA) or rabbit anti-Ym1 at a 1:100 dilution (Stem Cell, CA; Canada) overnight at 4 °C. Next, the slides were washed, with distilled water 3 times and mounted with one drop of Fluoroshield mounting medium with DAPI (Abcam; MA, USA) per tissue section. Immunofluorescence was analyzed using an Axio Vert A1 microscope (Zeiss).

4.7. ELISA

Peripheral blood was collected from the lateral tail vein and centrifuged at 2500 rpm for 10 min, and serum was collected and tested for *T. canis*-specific IgG1 and IgG2a in antigen-coated plates (1 µg/mL), excreted-secreted antigens of *T. canis* were used to sensitized plated were obtained according to previous protocols [68]. After overnight incubation at 4 °C, the plates were washed with PBS supplemented with 0.05% Tween 20 (Sigma; St. Louise, USA) and blocked with PBS supplemented with 1% BSA (Biowest). Serial dilutions of serum samples starting at 1:100 were added to the plates. Bound antibodies were detected following incubation with HRP-conjugated rat anti-mouse IgG1 or IgG2a (Zymed; San Francisco, USA) and read on a microplate reader at 405 nm (Multiskan Ascent, Thermo Labsystems). The results are expressed as the maximal sera dilution at which optical density (OD) was detected. Serum levels of IFN-γ, TNF-α, IL-4, IL-13, and IL-10 were measured by ELISA (Peprotech; Mexico City, Mexico) according to the manufacturer's instructions.

4.8. RT-PCR Analysis

Lungs were placed in 1.5 mL Eppendorf tubes containing TRIzol reagent (Invitrogen; CA, USA) and 0.5 mm diameter zirconium oxide beads (Next Advance) for tissue digestion in a bullet blender (Next Advance). RNA was extracted by the chloroform technique; once quantified, 1 µg of the product was reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen; CA, USA). The primers used to amplify genes and the melting temperatures are described in Table S1. Amplified products were mixed with loading buffer containing SYBR green and observed in a 1.5% agarose gel (IBI Scientific molecular biology certified) with a DocIM-EZ Gel Documentation System. Images were analyzed using ImageJ, and the expression values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a control (Table S1).

4.9. Flow Cytometry

Lungs obtained at 7 dpi were injected with saline solution to obtain cells. Red blood cells were lysed, and live cells were counted using trypan blue exclusion (Countess II FL ThermoFisher). The cells were stained for expression of surface markers using the following antibodies: anti-F4/80-Pacific blue or APC, anti-CD86-PE/Cy7, anti-CD206 (MMR)-PerCP or FITC and anti-IL4Rα-PE (all from Biolegend; CA, USA). The samples were incubated for 30 min at 4 °C in FACS sheet buffer (BD®). The samples were acquired using either a FACSAria Fusion (BD®) or Attune NxT (ThermoFisher) and analyzed using FlowJo software (version 10.0.7).

4.10. Macrophage Depletion with Clodronate Liposomes

We administered clodronate-loaded liposomes intratracheally according to the manufacturer's instructions (Formumax; CA, USA) to deplete macrophages in the lung tissue. Two days after liposome treatment, mice were infected as described. At day four after the first treatment, a second dose of 100 µL of clodronate-loaded or PBS-loaded liposomes was administered to the corresponding group. At 4 dpi, the animals were euthanized.

4.11. Statistical Analysis

Differences between groups were determined by either two-tailed unpaired Student's t-test or two-way analysis of variance (ANOVA), with multiple Holm–Sidak test, and reported as the means ± SEM. All statistical analyses were determined using GraphPad Prism v 6.0, with a "p" value or less than 0.05 considered significant (* $p < 0.05$). All experiments were carried out independently at least twice.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/8/4/280/s1, Figure S1: Collagen deposition, Figure S2: M1 and M2 markers after macrophages depletion, Figure S3: Numbers of cells in lung tissue, Table S1: Genes and their respective sequences used to determine macrophage and immune response activation.

Author Contributions: Conceptualization, B.F.-L. and L.I.T.; methodology, B.F.-L., I.M.-A. J.E.O. and J.P.L.; validation, B.F.-L., H.M.-R., R.H.-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; formal analysis, B.F.-L., H.M.-R., R.I.L-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; resources, R.I.L-P., D.M.M., J.E.O. and L.I.T.; data curation, B.F.-L., H.M.-R., R.H.-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; writing-original draft preparation, B.F.-L., H.M.-R., R.I.L-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; writing-review and editing, B.F.-L., H.M.-R., R.I.L-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; visualization, B.F.-L., H.M.-R., R.H.-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; supervision, B.F.-L., R.H.-P., D.M.M., J.E.O. and L.I.T.; project administration, B.F.-L., H.M.-R., R.H.-P., P.M.-L., D.M.M., I.M.-A., J.E.O. and L.I.T.; funding acquisition, L.I.T.

Funding: This research received no external funding.

Acknowledgments: Berenice Paz-López is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and has received CONACyT fellowship 515378. The authors thank MVZ Leticia Flores and Biol. Tomas Villamar for their support in the animal care facilities and to Emmanuel Molina-Guzman for his technical assistance in histology.

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

References

1. Ma, G.; Holland, C.V.; Wang, T.; Hofmann, A.; Fan, C.K.; Maizels, R.M.; Hotez, P.J.; Gasser, R.B. Human toxocariasis. *Lancet Infect. Dis.* **2018**, *18*, 14–24. [CrossRef]
2. Rostami, A.; Ma, G.; Wang, T.; Koehler, A.V.; Hofmann, A.; Chang, B.C.H.; Macpherson, C.N.; Gasser, R.B. Human toxocariasis—A look at a neglected disease through an epidemiological 'prism'. *Infect. Genet. Evol.* **2019**, *74*, 104002. [CrossRef] [PubMed]
3. Nagy, D.; Bede, O.; Danka, J.; Szenasi, Z.; Sipka, S. Analysis of serum cytokine levels in children with chronic cough associated with Toxocara canis infection. *Parasite Immunol.* **2012**, *34*, 581–588. [CrossRef] [PubMed]
4. Aghaei, S.; Riahi, S.M.; Rostami, A.; Mohammadzadeh, I.; Javanian, M.; Tohidi, E.; Foroutan, M.; Esmaeili Dooki, M. Toxocara spp. infection and risk of childhood asthma: A systematic review and meta-analysis. *Acta Trop.* **2018**, *182*, 298–304. [CrossRef]
5. Pinelli, E.; Brandes, S.; Dormans, J.; Fonville, M.; Hamilton, C.M.; der Giessen, J. Toxocara canis: Effect of inoculum size on pulmonary pathology and cytokine expression in BALB/c mice. *Exp. Parasitol.* **2007**, *115*, 76–82. [CrossRef]
6. Zaia, M.G.; Oliveira, S.R.; Castro, C.A.; Soares, E.G.; Afonso, A.; Monnazzi, L.G.; Peitl Filho, O.; Faccioli, L.H.; Anibal Fde, F. Toxocara canis and the allergic process. *Mem. Do Inst. Oswaldo Cruz* **2015**, *110*, 726–731. [CrossRef]
7. Martinez, F.O.; Gordon, S. The M1 and M2 paradigm of macrophage activation: Time for reassessment. *F1000prime Rep.* **2014**, *6*, 13. [CrossRef]
8. Wang, N.; Liang, H.; Zen, K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. *Front. Immunol.* **2014**, *5*, 614. [CrossRef]
9. Li, C.; Xu, M.M.; Wang, K.; Adler, A.J.; Vella, A.T.; Zhou, B. Macrophage polarization and meta-inflammation. *Transl. Res. J. Lab. Clin. Med.* **2018**, *191*, 29–44. [CrossRef]
10. Juhas, U.; Ryba-Stanislawowska, M.; Szargiej, P.; Mysliwska, J. Different pathways of macrophage activation and polarization. *Postep. Hig. Med. Dosw.* **2015**, *69*, 496–502. [CrossRef]
11. Reich, N.C. STATs get their move on. *Jak Stat* **2013**, *2*, e27080. [CrossRef] [PubMed]
12. Ruckerl, D.; Cook, P.C. Macrophages assemble! But do they need IL-4R during schistosomiasis? *Eur. J. Immunol.* **2019**, *49*, 996–1000. [CrossRef] [PubMed]
13. Gordon, S. Alternative activation of macrophages. *Nat. Rev. Immunol.* **2003**, *3*, 23–35. [CrossRef] [PubMed]
14. Minutti, C.M.; Jackson-Jones, L.H.; Garcia-Fojeda, B.; Knipper, J.A.; Sutherland, T.E.; Logan, N.; Ringqvist, E.; Guillamat-Prats, R.; Ferenbach, D.A.; Artigas, A.; et al. Local amplifiers of IL-4Ralpha-mediated macrophage activation promote repair in lung and liver. *Science* **2017**, *356*, 1076–1080. [CrossRef]
15. Vannella, K.M.; Wynn, T.A. Mechanisms of Organ Injury and Repair by Macrophages. *Annu. Rev. Physiol.* **2017**, *79*, 593–617. [CrossRef]
16. Wynn, T.A.; Vannella, K.M. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity* **2016**, *44*, 450–462. [CrossRef]
17. Kreider, T.; Anthony, R.M.; Urban, J.F., Jr.; Gause, W.C. Alternatively activated macrophages in helminth infections. *Curr. Opin. Immunol.* **2007**, *19*, 448–453. [CrossRef]
18. Faz-Lopez, B.; Morales-Montor, J.; Terrazas, L.I. Role of Macrophages in the Repair Process during the Tissue Migrating and Resident Helminth Infections. *BioMed Res. Int.* **2016**, *2016*, 8634603. [CrossRef]
19. Anthony, R.M.; Urban, J.F., Jr.; Alem, F.; Hamed, H.A.; Rozo, C.T.; Boucher, J.L.; Van Rooijen, N.; Gause, W.C. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat. Med.* **2006**, *12*, 955–960. [CrossRef]
20. Hung, L.Y.; Sen, D.; Oniskey, T.K.; Katzen, J.; Cohen, N.A.; Vaughan, A.E.; Nieves, W.; Urisman, A.; Beers, M.F.; Krummel, M.F.; et al. Herbert. Macrophages promote epithelial proliferation following infectious and non-infectious lung injury through a Trefoil factor 2-dependent mechanism. *Mucosal Immunol.* **2019**, *12*, 64–76. [CrossRef]
21. Jenkins, S.J.; Ruckerl, D.; Cook, P.C.; Jones, L.H.; Finkelman, F.D.; van Rooijen, N.; MacDonald, A.S.; Allen, J.E. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* **2011**, *332*, 1284–1288. [CrossRef] [PubMed]

22. He, X.; Tang, R.; Sun, Y.; Wang, Y.G.; Zhen, K.Y.; Zhang, D.M.; Pan, W.Q. MicroR-146 blocks the activation of M1 macrophage by targeting signal transducer and activator of transcription 1 in hepatic schistosomiasis. *EBioMedicine* 2016, *13*, 339–347. [CrossRef] [PubMed]
23. Nair, M.G.; Herbert, D.R. Immune polarization by hookworms: Taking cues from T helper type 2, type 2 innate lymphoid cells and alternatively activated macrophages. *Immunology* 2016, *148*, 115–124. [CrossRef] [PubMed]
24. Paz-Lopez, B.; Ledesma-Soto, Y.; Romero-Sanchez, Y.; Calleja, E.; Martinez-Labat, P.; Terrazas, L.I. Signal transducer and activator of transcription factor 6 signaling contributes to control host lung pathology but favors susceptibility against Toxocara canis infection. *BioMed Res. Int.* 2013, *2013*, 696343. [CrossRef] [PubMed]
25. Ikeda, N.; Asano, K.; Kikuchi, K.; Uchida, Y.; Ikegami, H.; Takagi, R.; Yotsumoto, S.; Shibuya, T.; Makino-Okamura, C.; Fukuyama, H.; et al. Emergence of immunoregulatory Ym1(+)Ly6C(hi) monocytes during recovery phase of tissue injury. *Sci. Immunol.* 2018, *3*. [CrossRef]
26. Franken, L.; Schiwon, M.; Kurts, C. Macrophages: Sentinels and regulators of the immune system. *Cell. Microbiol.* 2016, *18*, 475–487. [CrossRef]
27. Shapouri-Moghaddam, A.; Mohammadian, S.; Vazini, H.; Taghadosi, M.; Esmaeili, S.A.; Mardani, F.; Seifi, B.; Mohammadi, A.; Afshari, J.T.; Sahebkar, A. Macrophage plasticity, polarization, and function in health and disease. *J. Cell. Physiol.* 2018, *233*, 6425–6440. [CrossRef]
28. Gordon, S.; Pluddemann, A.; Martinez Estrada, F. Macrophage heterogeneity in tissues: Phenotypic diversity and functions. *Immunol. Rev.* 2014, *262*, 36–55. [CrossRef]
29. Porta, C.; Riboldi, E.; Ippolito, A.; Sica, A. Molecular and epigenetic basis of macrophage polarized activation. *Semin. Immunol.* 2015, *27*, 237–248. [CrossRef]
30. Schultze, J.L.; Schmidt, S.V. Molecular features of macrophage activation. *Semin. Immunol.* 2015, *27*, 416–423. [CrossRef]
31. Noel, W.; Raes, G.; Hassanzadeh Ghassabeh, G.; De Baetselier, P.; Beschin, A. Alternatively activated macrophages during parasite infections. *Trends Parasitol.* 2004, *20*, 126–133. [CrossRef] [PubMed]
32. Mosser, D.M.; Edwards, J.P. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 2008, *8*, 958–969. [CrossRef] [PubMed]
33. Xue, J.; Schmidt, S.V.; Sander, J.; Draffehn, A.; Krebs, W.; Quester, I.; De Nardo, D.; Gohel, T.D.; Emde, M.; Schmidleithner, L.; et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 2014, *40*, 274–288. [CrossRef] [PubMed]
34. Reyes, J.L.; Terrazas, L.I. The divergent roles of alternatively activated macrophages in helminthic infections. *Parasite Immunol.* 2007, *29*, 609–619. [CrossRef]
35. Mantovani, A.; Sica, A.; Sozzani, S.; Allavena, P.; Vecchi, A.; Locati, M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 2004, *25*, 677–686. [CrossRef]
36. Murray, P.J.; Allen, J.E.; Biswas, S.K.; Fisher, E.A.; Gilroy, D.W.; Goerdt, S.; Gordon, S.; Hamilton, J.A.; Ivashkiv, L.B.; Lawrence, T.; et al. Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity* 2014, *41*, 14–20. [CrossRef]
37. Gause, W.C.; Wynn, T.A.; Allen, J.E. Type 2 immunity and wound healing: Evolutionary refinement of adaptive immunity by helminths. *Nat. Rev. Immunol.* 2013, *13*, 607–614. [CrossRef]
38. Allen, J.E.; Sutherland, T.E. Host protective roles of type 2 immunity: Parasite killing and tissue repair, flip sides of the same coin. *Semin. Immunol.* 2014, *26*, 329–340. [CrossRef]
39. Aranzamendi, C.; Sofronic-Milosavljevic, L.; Pinelli, E. Helminths: Immunoregulation and Inflammatory Diseases-Which Side Are *Trichinella* spp. and *Toxocara* spp. on? *J. Parasitol. Res.* 2013, *2013*, 329438. [CrossRef]
40. Maizels, R.M. Toxocara canis: Molecular basis of immune recognition and evasion. *Vet. Parasitol.* 2013, *193*, 365–374. [CrossRef]
41. Sutherland, T.E.; Ruckerl, D.; Logan, N.; Duncan, S.; Wynn, T.A.; Allen, J.E. Ym1 induces RELMalpha and rescues IL-4Ralpha deficiency in lung repair during nematode infection. *PLoS Pathog.* 2018, *14*, e1007423. [CrossRef] [PubMed]
42. Vergadi, E.; Ieronymaki, E.; Lyroni, K.; Vaporidi, 80 Tsatsanis, C. Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization. *J. Immunol.* 2017, *198*, 1006–1014. [CrossRef] [PubMed]

43. O'Farrell, A.M.; Liu, Y.; Moore, K.W.; Mui, A.L. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: Evidence for Stat3-dependent and -independent pathways. *EMBO J.* **1998**, *17*, 1006–1018. [CrossRef] [PubMed]
44. Bando, J.K.; Nussbaum, J.C.; Liang, H.E.; Locksley, R.M. Type 2 innate lymphoid cells constitutively express arginase-I in the naive and inflamed lung. *J. Leukoc. Biol.* **2013**, *94*, 877–884. [CrossRef]
45. Turner, J.E.; Morrison, P.J.; Wilhelm, C.; Wilson, M.; Ahlfors, H.; Renauld, J.C.; Panzer, U.; Helmby, H.; Stockinger, B. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J. Exp. Med.* **2013**, *210*, 2951–2965. [CrossRef]
46. Sonnenberg, G.F.; Artis, D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. *Nat. Med.* **2015**, *21*, 698–708. [CrossRef]
47. Reece, J.J.; Siracusa, M.C.; Southard, T.L.; Brayton, C.F.; Urban, J.F., Jr.; Scott, A.L. Hookworm-induced persistent changes to the immunological environment of the lung. *Infect. Immun.* **2008**, *76*, 3511–3524. [CrossRef]
48. Loser, S.; Smith, K.A.; Maizels, R.M. Innate Lymphoid Cells in Helminth Infections-Obligatory or Accessory? *Front. Immunol.* **2019**, *10*, 620. [CrossRef]
49. Gieseck, R.L., III; Wilson, M.S.; Wynn, T.A. Type 2 immunity in tissue repair and fibrosis. *Nat. Rev. Immunol.* **2018**, *18*, 62–76. [CrossRef]
50. Gazzinelli-Guimaraes, P.H.; Nutman, T.B. Helminth parasites and immune regulation. *F1000Research* **2018**, *7*. [CrossRef]
51. Jimenez, P.A.; Jimenez, S.E. Tissue and cellular approaches to wound repair. *Am. J. Surg.* **2004**, *187*, 56–64. [CrossRef]
52. Soldano, S.; Pizzorni, C.; Paolino, S.; Trombetta, A.C.; Montagna, P.; Brizzolara, R.; Ruaro, B.; Sulli, A.; Cutolo, M. Alternatively Activated (M2) Macrophage Phenotype Is Inducible by Endothelin-1 in Cultured Human Macrophages. *PLoS ONE* **2016**, *11*, e0166433. [CrossRef] [PubMed]
53. Hendrix, A.Y.; Kheradmand, F. The Role of Matrix Metalloproteinases in Development, Repair, and Destruction of the Lungs. *Prog. Mol. Biol. Trans. Sci.* **2017**, *148*, 1–29. [CrossRef]
54. Reddel, C.J.; Weiss, A.S.; Burgess, J.K. Elastin in asthma. *Pulm. Pharmacol. Ther.* **2012**, *25*, 144–153. [CrossRef]
55. Pfaff, A.W.; Schulz-Key, H.; Soboslay, P.T.; Geiger, S.M.; Hoffmann, W.H. The role of nitric oxide in the innate resistance to microfilariae of Litomosoides sigmodontis in mice. *Parasite Immunol.* **2000**, *22*, 397–405. [CrossRef]
56. Rodrigues, R.M.; Goncalves, A.L.R.; Silva, N.M.; Cardoso, C.R.B.; Araujo, N.R.; Coutinho, L.B.; Alves, R.; Ueta, M.T.; Costa-Cruz, J.M. Inducible nitric oxide synthase controls experimental Strongyloides infection. *Parasite Immunol.* **2018**, *40*, 12576. [CrossRef]
57. Pesce, J.T.; Ramalingam, T.R.; Wilson, M.S.; Mentink-Kane, M.M.; Thompson, R.W.; Cheever, A.W.; Urban, J.F., Jr.; Wynn, T.A. Retnla (relmalpha/fizz1) suppresses helminth-induced Th2-type immunity. *PLoS Pathog.* **2009**, *5*, e1000393. [CrossRef]
58. Filbey, K.J.; Camberis, M.; Chandler, J.; Turner, R.; Kettle, A.J.; Eichenberger, R.M.; Giacomin, P.; Gros, G.L. Intestinal helminth infection promotes IL-5-and CD4(+) T cell-dependent immunity in the lung against migrating parasites. *Mucosal Immunol.* **2019**, *12*, 352–362. [CrossRef]
59. Esser-von Bieren, J.; Mosconi, I.; Guiet, R.; Piersgilli, A.; Volpe, B.; Chen, F.; Gause, W.C.; Seitz, A.; Verbeek, J.S.; Harris, N.L. Antibodies trap tissue migrating helminth larvae and prevent tissue damage by driving IL-4Ralpha-independent alternative differentiation of macrophages. *PLoS Pathog.* **2013**, *9*, e1003771. [CrossRef]
60. Masure, D.; Vlaminck, J.; Wang, T.; Chiers, K.; Van den Broeck, W.; Vercruysse, J.; Geldhof, P. A role for eosinophils in the intestinal immunity against infective Ascaris suum larvae. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2138. [CrossRef]
61. Galioto, A.M.; Hess, J.A.; Nolan, T.J.; Schad, G.A.; Lee, J.J.; Abraham, D. Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval strongyloides stercoralis in mice. *Infect. Immun.* **2006**, *74*, 5730–5738. [CrossRef] [PubMed]
62. Holmes, D.A.; Yeh, J.H.; Yan, D.; Xu, M.; Chan, A.C. Dusp5 negatively regulates IL-33-mediated eosinophil survival and function. *EMBO J.* **2015**, *34*, 218–235. [CrossRef] [PubMed]

63. Rockey, J.H.; John, T.; Donnelly, J.J.; McKenzie, D.F.; Stromberg, B.E.; Soulsby, E.J. In vitro interaction of eosinophils from ascarid-infected eyes with *Ascaris suum* and *Toxocara canis* larvae. *Investig. Ophthalmol. Vis. Sci.* 1983, *24*, 1346–1357.
64. Takamoto, M.; Ovington, K.S.; Behm, C.A.; Sugane, K.; Young, I.G.; Matthaei, K.I. Eosinophilia, parasite burden and lung damage in Toxocara canis infection in C57Bl/6 mice genetically deficient in IL-5. *Immunology* **1997**, *90*, 511–517. [CrossRef] [PubMed]
65. Rodolpho, J.M.A.; Camillo, L.; Araujo, M.S.S.; Speziali, E.; Coelho-Dos-Reis, J.G.; Correia, R.O.; Neris, D.M.; Martins-Filho, O.A.; Teixeira-Carvalho, A.; Anibal, F.F. Robust Phenotypic Activation of Eosinophils during Experimental *Toxocara canis* Infection. *Front. Immunol.* 2018, *9*, 64. [CrossRef]
66. Ariyaratne, A.; Finney, C.A. Eosinophils and Macrophages within the Th2-Induced Granuloma: Balancing Killing and Healing in a Tight Space. *Infect. Immun.* **2019**, *87*. [CrossRef]
67. Alba-Hurtado, F.; Munoz-Guzman, M.A.; Valdivia-Anda, G.; Tortora, J.L.; Ortega-Pierres, M.G. Toxocara canis: Larval migration dynamics, detection of antibody reactivity to larval excretory-secretory antigens and clinical findings during experimental infection of gerbils (*Meriones unguiculatus*). *Exp. Parasitol.* **2009**, *122*, 1–5. [CrossRef]
68. Watthanakulpanich, D.; Smith, H.V.; Hobbs, G.; Whalley, A.J.; Billington, D. Application of Toxocara canis excretory-secretory antigens and IgG subclass antibodies (IgG1-4) in serodiagnostic assays of human toxocariasis. *Acta Trop.* **2008**, *106*, 90–95. [CrossRef]

SCIENTIFIC REPORTS

OPEN

Suppression of colitis by adoptive transfer of helminth antigen-treated dendritic cells requires interleukin-4 receptor-α signaling

Received: 03 October 2016
Accepted: 00 December 2016
Published: 17 January 2017

C. E. Matisz[1], B. Faz-López[2], E. Thomson[1], A. Al Rajabi[1], F. Lopes[1], L. I. Terrazas[2], A. Wang[1], K. A. Sharkey[1,3] & D. M. McKay[1]

Infection with helminth parasites has been explored as a treatment for autoimmune and inflammatory diseases. As helminth antigens have potent immunomodulation properties capable of inducing regulatory programs in a variety of cell types, transferring cells treated with helminth antigens represents a novel extension to helminth therapy. Previous work determined that transfer of bone marrow-derived dendritic cells (DC) pulsed with a crude extract of the tapeworm *Hymenolepis diminuta* (HD) can suppress colitis in recipient mice. The present study explored the mechanism of disease suppression and the importance of interleukin (IL)-4 signaling. Transfer of HD-DCs suppressed dinitrobenzene sulfonic acid (DNBS)-induced colitis through activation of recipient IL-4 receptor-α. The transferred HD-DCs required IL-4Rα and the capacity to secrete IL-10 to drive IL-4 and IL-10 production and to suppress colitis in recipient mice. Treatment of DCs with IL-4 evokes an alternatively activated phenotype, but adoptive transfer of these cells did not affect the outcome of colitis. Collectively, these studies demonstrate the complexity between IL-4 and IL-10 in donor cells and recipient, and the requirement for parasite- and host-derived factors in this novel form of cell therapy. Thus IL-4Rα signaling is revealed as a pathway that could be exploited for helminth antigen cell-based therapy.

Epidemiological studies that highlight the inverse relationship between exposure to helminth parasites and the incidence of inflammatory disease, combined with the known immunomodulatory capacity of helminths, have led to a renewed interest in the "therapeutic helminth"[1–3]. Analyses of murine models of disease, patient case reports, and small clinic trials have illustrated the suppression of inflammatory disease by infection with helminth parasites[4–6]. As an extension of this approach, systemic administration of antigens and secreted molecules from a variety of helminth parasites has been used to inhibit disease in rodent model systems[7–10]. A more recent aspect of helminth therapy is the prospect of using immune cells pulsed with or educated by exposure to helminth antigens[11,12]. Cellular immunotherapy, with hematopoietic or mesenchymal stem cells, is used to treat a variety of diseases[13–15], and is being considered as an approach to treat inflammatory bowel disease, notably Crohn's disease[15–19].

We have recently shown that adoptive transfer of dendritic cells (DC) treated with a somatic extract of the adult rat tapeworm, *Hymenolepis diminuta* (HD) (HD-DCs), significantly attenuated the severity of dinitrobenzene sulphonic acid (DNBS) induced colitis in mice. The suppression of disease in the HD-DC treated mice was dependent on mobilization of adaptive immunity and was associated with the capacity of splenocytes in the recipients to produce IL-10 and IL-4[20]. While a role for IL-10 in the anti-colitic effect was defined, the significance, if any, of the increased production of IL-4 in the context of the biology of the DC was not addressed. Thus, this was the focus of the present study.

It has been shown that IL-4 converts DCs to an alternatively activated phenotype (AADC)[21], somewhat analogous to the IL-4-induced alternatively activated macrophage (AAM)[22], but little is known of the function of the

[1]Gastrointestinal Research Group and Inflammation Research Network, Department of Physiology and Pharmacology, Calvin, Joan and Phoebe Snyder Institute for Chronic Diseases, Cumming School of Medicine, University of Calgary, Canada. [2]Unidad de Biomedicina, FES Iztacala, Universidad Nacional Autónoma de México, Estado de México, México. [3]Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada. Correspondence and requests for materials should be addressed to D.M.M. (email: dmckay@ucalgary.ca)

former cell type. In the present study, using IL-4 receptor-α knock-out mice as donors or recipients of HD-DCs revealed that the anti colitic effect of these cells required IL-4Rα on both the transferred cell and in the recipient mice. However, adoptive transfer of IL-4-induced AADC had no impact on the outcome of DNBS-induced colitis. Thus, the combination of exposure to an extract of *H. diminuta* and IL-4 generates a regulatory DC that can suppress colitis. These findings demonstrate the importance of IL-4Rα signaling in helminth antigen-pulsed DCs, and highlight a novel pathway that could be exploited for the use of helminth-antigen cell-based therapy.

Results

Attenuation of colitis by HD-DCs is positively correlated with splenic IL-4. Adoptive transfer of HD-DCs attenuates the severity of DNBS-induced colitis and this is accompanied by increased production of IL-4 by mitogen-stimulated splenocytes from the recipient mice[20]. Correlation analysis revealed a significant relationship between splenocyte IL-4 production and colitis disease activity score, such that those mice treated with HD-DCs that produced more IL-4 had less severe colitis (Fig. 1A). A trend towards a negative correlation between IL-10 and disease severity (Fig. 1B), and a positive correlation between splenic IL-4 and IL-10 production (Fig. 1C) was also observed. Thus, a role for IL-4 in the anti colitic effect conferred by adoptive transfer of HD-DCs was tested.

Recipients require IL-4Rα signaling for HD-DC suppression of colitis. Adoptive transfer of wild-type (WT) HD-DCs into normal mice significantly suppressed DNBS-induced colitis, as before[20], but this was not observed in recipient IL-4Rα$^{-/-}$ mice (Fig. 2A–C). In accordance with these data, splenocytes from WT mice given HD-DCs challenged with DNBS showed increased IL-4 and IL-10 output from stimulated splenocytes compared to time-matched IL-4Rα$^{-/-}$ mice (Fig. 2D,E). Administration of HD-DCs to IL-4Rα$^{-/-}$ mice that did not get DNBS also resulted in less IL-4 output from splenic T cells stimulated 5 days after receiving the cells compared to WT mice [Mean ± SEM of IL-4: WT recipients = 2188 ± 87 pg/mL; IL4Rα$^{-/-}$ recipients = 1485 ± 165 pg/mL; $p = 0.0198$, $n = 3$]. Thus, an IL-4 feedback loop exists that is important for the anti-colitic effect of HD-DCs, such that these cells stimulate IL-4 production, which is important for the anti colitic effect since the HD DCs are ineffective in IL-4Rα$^{-/-}$ mice (Fig. 2A–C).

HD-DCs require IL-4Rα to suppress DNBS colitis in recipients. Considering the possibility that IL-4 signaling in the dendritic cell could be important for its anti-colitic activity, WT HD-DCs and IL-4Rα$^{-/-}$ HD-DCs were transferred to WT mice, 48 hours prior to DNBS challenge. Mice that received WT HD DC, but not IL-4Rα$^{-/-}$ HD-DC, displayed significantly attenuated macroscopic damage (Fig. 3A), and reduced histological damage (Fig. 3B,C). The absence of IL-4Rα completely abrogated the ability of HD DCs to drive splenic IL-4 and IL-10 in recipients (Fig. 3D,E). It has been reported that IL-4 can induce IL-4 production by DCs[23]. However, treatment with IL-4 resulted in similar levels of IL-4 in the supernatants of WT and IL4Rα$^{-/-}$ DCs, and co-treatment with HD antigen did not significantly enhance the ability of IL-4 to induce DC IL-4 synthesis (Fig. 3F). In addition, IL-4 mRNA was not detected in DCs treated with HD antigen, IL-4, or in combination.

HD-DCs induce a population of IL-4 producing CD4$^+$ and CD19$^+$ splenocytes. To assess the source of splenic IL-4, mice were administered DCs or HD-DCs, and 5 days later splenocytes were counted and stained for IL-4 (Fig. 4A). Adoptive transfer of HD-DC significantly increased the proportion of IL-4 secreting CD4$^+$ and CD19$^+$ splenocytes in recipient mice (Fig. 4B,D). No differences in CD8$^+$ IL-4$^+$ cells were observed (Fig. 4C).

IL-10$^{-/-}$ HD-DCs do not suppress DNBS colitis. IL-10 producing DCs have been demonstrated to drive IL-4 production in CD4$^+$ cells *in vitro*[24], and induce tolerogenic T cell populations[25]. Thus the importance of IL-10 secretion by adoptively transferred HD-DCs was considered. WT and IL-10$^{-/-}$ HD-DCs were transferred to WT mice, 48h prior to DNBS challenge. Contrary to WT HD DCs, IL-10$^{-/-}$ HD DCs were unable to attenuate the severity of colitis (Fig. 5A–C), nor were they able to drive splenic IL-4 or IL-10 in recipients (Fig. 5D,E). Levels of transcript and secreted IL-10 were below level of detection in HD DC treated cells *in vitro*.

IL-4 alternatively activated DCs do not suppress DNBS-induced colitis. IL-4 treated macrophages take on an alternatively activated phenotype (AAMs) characterized by expression of arginase1, Ym1, and Relm α[22], and the adoptive transfer of IL-4 AAMs can suppress DNBS-induced colitis[26]. Similarly, analysis of Ym1 and Relm α expression reveals that IL-4 causes alternative activation in DCs (AADCs)[21], raising the possibility that HD antigen was, through its induction of IL-4, inducing an AADC phenotype that could block colitis. Replicating the findings of Cook *et al.*, IL-4 increased Relm α transcript and Ym1 and transcript (i.e. chil 3) and protein expression in DC, and we find that arginase-1 mRNA and protein are also up-regulated (Fig. 6A,B). HD antigen alone did not affect the expression of any of these markers, nor did it modulate the effect of IL-4 with the exception of suppression of Relm-α mRNA expression (Fig. 6A,B).

In contrast to HD-DCs, adoptive transfer of IL-4 AADCs did not affect the outcome of DNBS-induced colitis as assessed by macroscopic and histopathology damage scores (Fig. 6C–E), and did not induce IL-4 or IL-10 in the spleen (Fig. 6E,G). Adoptive transfer of DCs treated with HD+IL-4 attenuated DNBS colitis by ~35%, a degree of suppression similar to ~41% observed with HD-DCs as assessed by disease activity score (DNBS = 13.7 ± 0.9, $n = 3$; HD-DC+DNBS = 8.2 ± 3.1, $n = 3$; HD+IL-4-DC+DNBS = 9.0 ± 1.0, $n = 4$; mean ± SEM).

HD modulates IL-4 enhanced LPS pro-inflammatory responses. IL-4 not only elicits an AADC phenotype, it can also promote pro-inflammatory responses in DCs *in vitro*[27,28]. Since TNBS-induced colitis is accompanied by increased serum levels of LPS[29], it is likely that transferred cells are exposed to LPS in DNBS challenged mice. To mimic this putative *in vivo* interaction, DCs were treated with HD antigen for 24 h, and then stimulated with IL-4 and LPS for an additional 24 h. Levels of IL-12 (p40) and IL-10 produced by DCs treated

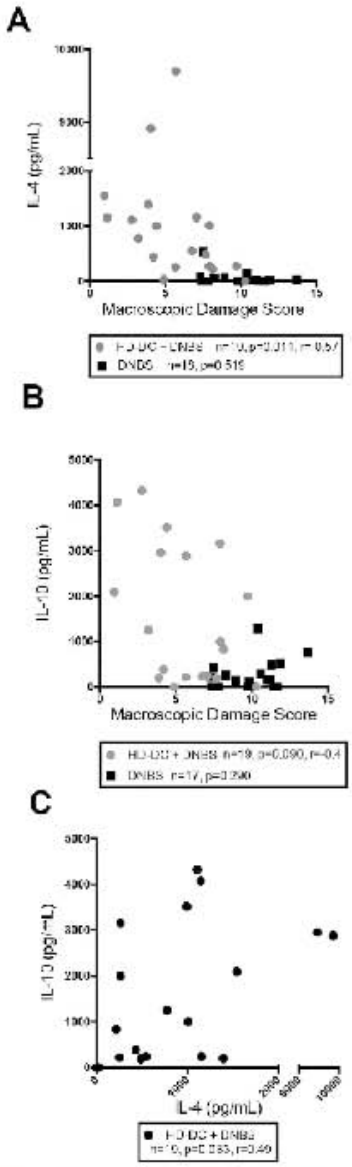

Figure 1. Suppression of colitis by HD-DC transfer is correlated with splenic IL-4 and IL-10 production. Individual macroscopic disease scores of mice challenged with DNBS, some of which had received 10^6 HD DCs i.p. 48 h prior, were plotted alongside their levels of splenic IL-4 (A) IL-10 (B). Splenic IL-4 and IL-10 in recipients of HD-DCs were plotted against each other in (C). Cytokine levels were measured by ELISA from the supernatants of conA (2 μg/mL) stimulated splenocytes (5×10^6/mL). As a D'Agostino & Pearson omnibus normality test determined cytokine data was not normally distributed; Spearman correlation test was performed for all graphs. Data from 4 independent experiments.

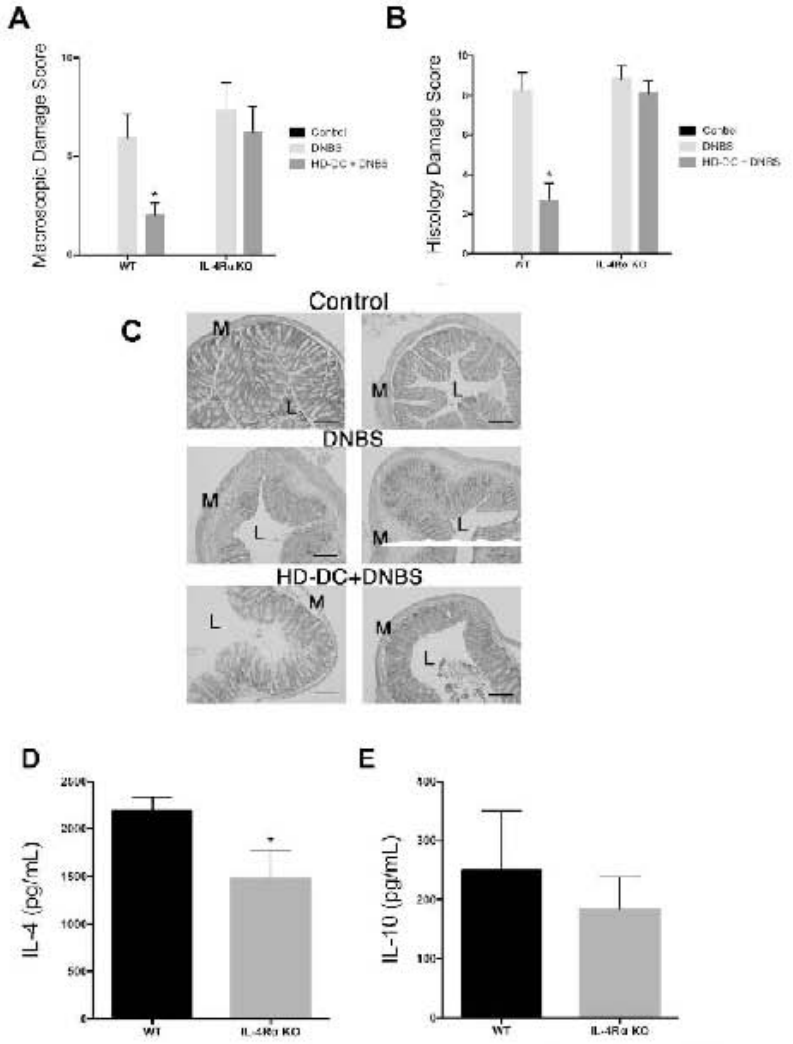

Figure 2. HD DCs do not suppress DNBS in IL-4Rα$^{-/-}$ mice. In house bred wild type (WT) or IL-4Rα$^{-/-}$ on a Balb/c background were given 10^6 HD DC ip. 48 h prior to DNBS (3 mg, i.r.) challenge. Mice were necropsied and assessed 72 h later via (A) macroscopic damage score and (B) histological damage score; (C) representative micrographs of H&E stained cross sections of colonic tissue: L: lumen; M: muscle; scale bar 500 μm. Splenocytes were isolated (5×10^6), stimulated with conA (2 μg/mL, 48 h) and supernatants examined for levels of IL-4 (D) and IL-10 (E) by ELISA. Data are shown as mean ± SEM; A–C are representative from one of two independent experiments, (*$p < 0.05$ vs. DNBS group, Kruskal-Wallis with Dunn's post-test, n = 3–6 mice/group) and D,E are pooled from two independent experiments (n = 7–9; two-tailed unpaired Student's t-test).

with IL-4, HD antigen alone, or in combination were below detection by ELISA. Treatment with HD antigen significantly suppressed LPS-induced IL-12 production by approximately 30%, while DCs treated with HD and IL-4 exhibited a 65% reduction in LPS induced IL-12 (Fig. 7A; LPS 4.6 ± 0.8; HD+LPS 3.3 ± 0.7; IL-4+LPS 2.7 ± 0.6; HD+IL-4+LPS 1.6 ± 0.3 IL-12p40 ng/mL). Only DCs treated with HD antigen in the absence of IL-4 displayed

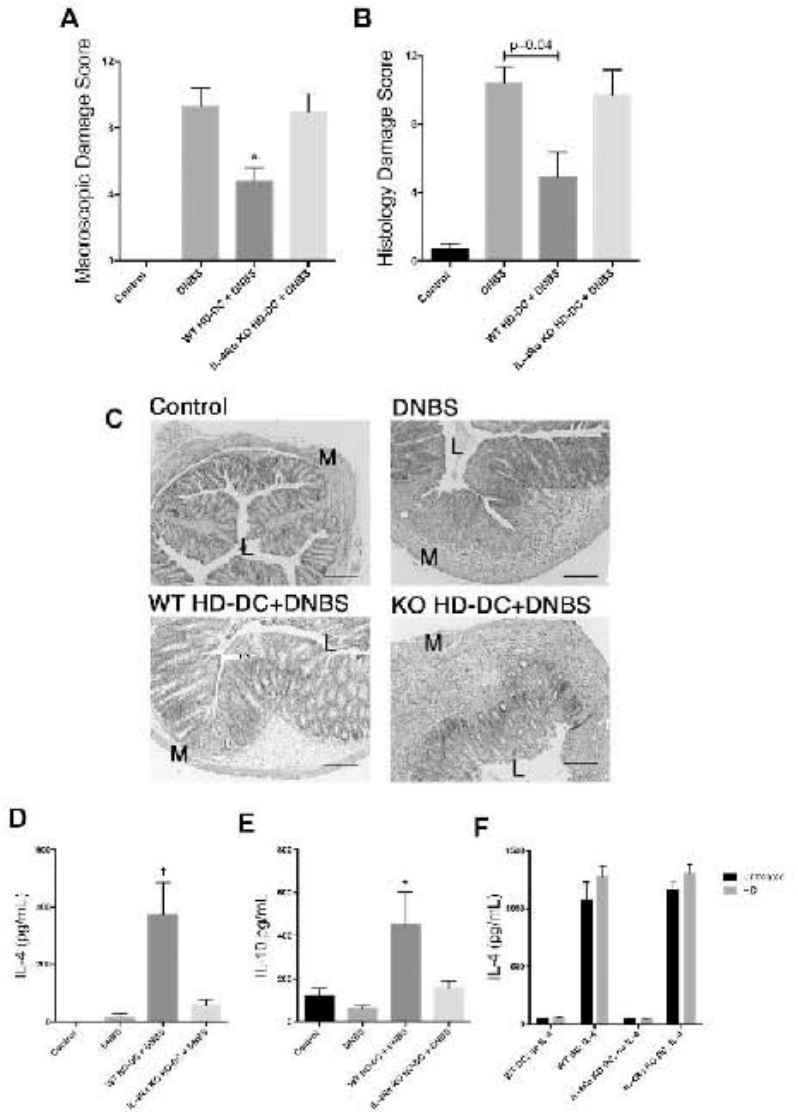

Figure 3. HD-DCs require IL-4Rα to suppress DNBS colitis in recipients. Balb/c mice were given 10^6 wild type or IL-4Rα$^{-/-}$ HD-DC ip. 48 h prior to DNBS (3 mg, in.) challenge. Mice were necropsied and assessed 72 h later via (A) macroscopic damage score (Kruskal Wallis with Dunn's post test $n = 6$–10 mice/group from 2 experiments) and (B) histological damage score (Mann-Whitney $n = 6$–10 mice/group from 2 experiments); (C) representative micrographs of H&E stained cross sections of colonic tissue: L: lumen; M: muscle; scale bar 300 μm. (D,E) Splenocytes were isolated (5×10^6), stimulated with conA (2 μg/mL, 48 h) and supernatants examined for levels of IL-4 and IL-10 ($n = 6$–10 mice/group from 2 experiments) by ELISA, and analyzed by one way ANOVA with Tukey's post test. (F) WT and IL-4Rα$^{-/-}$ DCs were treated with HD antigen (100 μg/mL) ± IL-4 (20 ng/mL); $n = 4$ from 1 experiment; one-way ANOVA with Sidak's multiple comparison among selected groups. All data are shown as mean ± SEM; $p \leq 0.05$ *compared to DNBS, †compared to all groups.

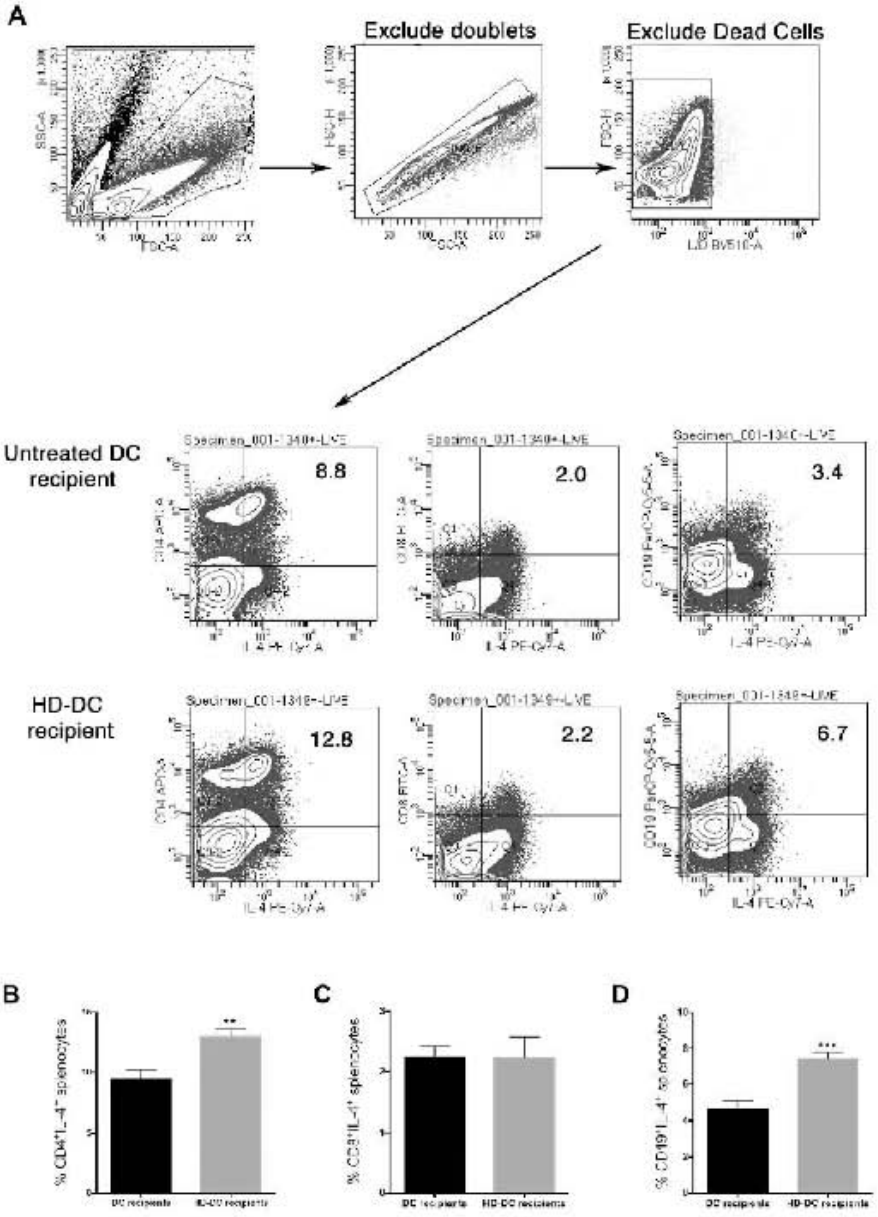

Figure 4. HD DCs induce IL-4 producing CD4⁺ and CD19⁺ splenocytes in recipient mice. Untreated DC or HD DCs (10^6) were adoptively transferred into Balb/c mice. Five days later, total splenocytes were counted, and following fixation and permeabilization, splenocytes were stained and analyzed by flow cytometry. Complete gating strategy is depicted in (A). The proportion of IL-4 positive cells was assessed in $CD4^+$ (B), $CD8^+$ (C), and $CD19^+$ (D) splenocytes. All data are shown as mean ± SEM; n = 5–6 from one of two representative experiments; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$., unpaired two tailed Student's t test.

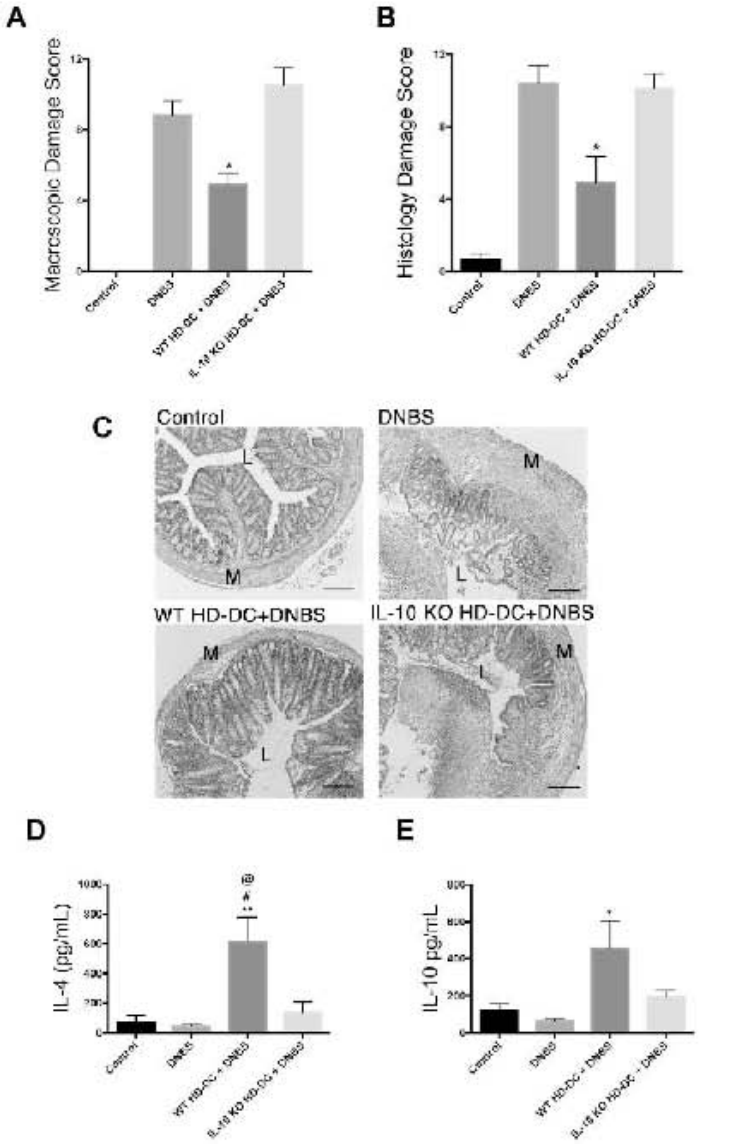

Figure 5. IL-10 KO HD-DCs cannot suppress colitis. Balb/c mice were given 10^6 wild-type or IL-$10^{-/-}$ HD-DC i.p. 48 h prior to DNBS (3 mg, i.r.) challenge. Mice were necropsied and assessed 72 h later via (**A**) macroscopic damage score and (**B**) histological damage score using Kruskal-Wallis with Dunn's post-test. (**C**) Representative micrographs of H&E stained cross-sections of colonic tissue; *L*: lumen; *M*: muscle; scale bar 300 μm. Splenocytes were isolated (5×10^6), stimulated with conA (2 μg/ml, 48 h) and supernatants examined for levels of IL-4 (**D**) and IL-10 (**E**) by ELISA, and analyzed by one-way ANOVA with Tukey's post-test. Data are shown as mean ± SEM and are pooled from two independent experiments; n = 6–10 mice/group; $^{*}p \leq 0.05$, $^{**}p < 0.01$ compared to DNBS, # compared to control, @ compared to IL-10 KO HD-DCs.

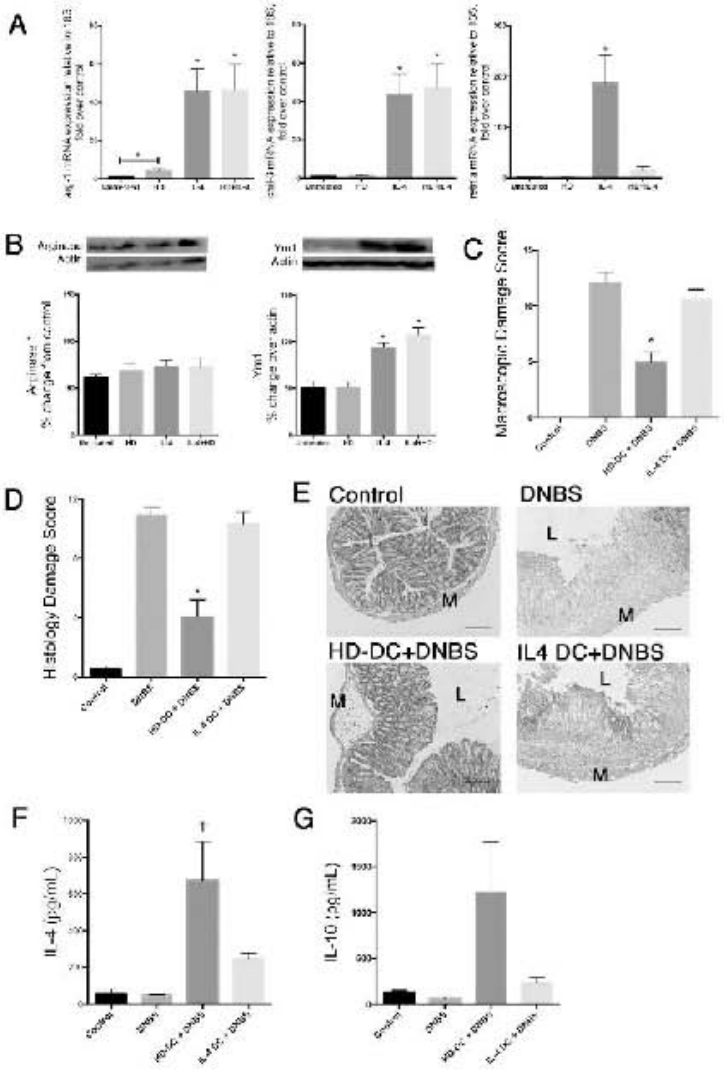

Figure 6. IL-4 alternatively activated DCs are not able to suppress colitis in recipients. Levels of arg-1, chil3 (Ym1), and retnla mRNA expression (A; n = 6–10 from 3 experiments) and arginase-1 and Ym1 protein expression (B; n = 6 from 2 experiments), with representative western blots, in untreated DCs, and DCs treated with HD antigen (100 µg/mL) and/or IL-4 (20 ng/mL) for 24 h. Data are shown as mean ± SEM; Repeated one-way ANOVA with Dunn's post-test; $^{*}p \leq 0.05$. For additional experiments, Balb/c mice were given 10^6 HD DC (100 µg/mL) or IL-4 alternatively activated (AA) DCs (20 ng/mL) ip, 48 h prior to DNBS (3 mg, i.r.) challenge. Mice were necropsied and assessed 72 h later via (C) macroscopic damage score and (D) histological damage score, with Kruskal-Wallis and Dunn's post-test. (E) Representative micrographs of H&E stained cross-sections of colonic tissue: L: lumen; M: muscle; scale bar 300 µm). Splenocytes were isolated (5×10^6), stimulated with conA (2 µg/mL, 48 h) and supernatants examined for levels of IL-4 (F) and IL-10 (G) by ELISA, and analyzed by one-way ANOVA with Tukey's post-test. Data are shown as mean + SEM and are pooled from two independent experiments; n = 6–8 mice/group; $p < 0.05$: *compared to DNBS, †compared to all groups.

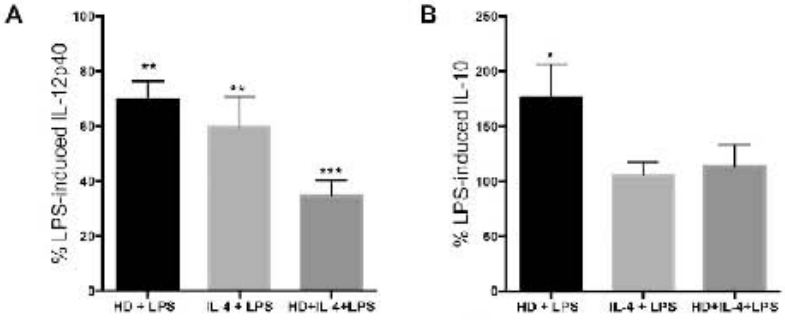

Figure 7. IL-12 and IL-10 production by HD-DCs exposed to LPS and IL-4. Dendritic cells were treated with HD antigen (100 μg/mL) 24 h prior to treatment with IL-4 (20 ng/mL) and LPS (1 μg/mL). Levels of IL-12p40 and IL-10 were assessed in the supernatants by ELISA 24 hours post stimulation of BMDCs with LPS and depicted as proportions of LPS-induced IL-12 (A; n = 8 from 2 experiments) and IL-10 (B; n = 4 from 1 experiment). Data are shown as mean ± SEM; one sample, two tailed *t* test to hypothetical mean of 100; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

significant increases (175%) in LPS-induced IL-10 (Fig. 7B; LPS 250 ± 11; HD + LPS 443 ± 46; IL-4 + LPS 264 ± 13; HD+IL 4+LPS 283 ± 16 IL 10 pg/mL). IL 4 enhanced LPS induced IL 12 production only when added 24 h prior to LPS challenge, and this too was attenuated by HD (mean ± SEM of IL-12p40: LPS 2484 ± 449; LPS+ IL 4 = 5310 ± 1106*; HD+LPS = 1758 ± 487; HD+IL 4+LPS = 2611 ± 731 pg/mL; $p < 0.05$; n = 11).

Discussion

Helminth therapy has been presented as a novel and natural way to inhibit auto-inflammatory diseases[48]. These are heterogeneous disorders and so it is reasonable to speculate that effective helminth therapy would be equally heterogeneous, requiring a specific helminth, a helminth extract or purified bioactive molecule, or autologous transplantation using the patients' own cells pulsed with a specific helminth extract: these approaches would mobilize a diverse array of anti inflammatory mechanisms[33]. Adoptive transfer of HD DCs suppresses DNBS-induced colitis, with the greatest benefit occurring in those mice showing significant increases in IL-10 and IL-4 production by splenic T cells[20]. Interleukin-10 is a prototypic anti-inflammatory cytokine and having defined its involvement in HD DC suppression of colitis[20], we focused on the putative role of IL-4. The data obtained indicate that IL-4Rα – and by inference IL-4 signaling – is required in both recipient and donor for HD DCs to block DNBS induced colitis.

Infection with helminth parasites elicits IL-4 production in mammalian hosts from a variety of cells[31–33], with T cells activated by helminth educated DCs being a major source of the cytokine[31,36]. For instance, mice infected with *H. diminuta* have increased synthesis of IL-4[37,38], and the demonstration that adoptive transfer of HD-DCs promotes splenocyte production of IL 4 upon reactivation *in vitro* is in accordance with the data from use of the parasite. Both $CD4^+$ T cells and $CD19^+$ B cells were identified as sources of IL 4 in mice receiving HD DCs; the latter being noteworthy given the demonstration of an anti-colitic regulatory B cell in *H. diminuta*-infected mice[39], and generation of an IL 4 producing B cell that was dependent on T cells and IL 4Rα in mice infected with the nematode parasite *Heligosomoides polygyrus*[40]. Furthermore, the observation that functional IL-4Rα in recipient mice is required for the anti colitic effect of HD DCs is consistent with data showing mice lacking signal transducer and activator of transcription (STAT)-6, the major intracellular signaling pathway from the IL-4 receptor, are not protected from DNBS-induced colitis by infection with *H. diminuta*[7].

A number of possibilities exist for the mechanism of action of IL-4Rα signaling in the suppression of colitis following adoptive transfer of HD-DCs. IL-4 is the prototypic TH2 cytokine perpetuating TH2 immunity[41,42]. It can exert anti colitic effects, as shown by adenoviral delivery or liposome encapsulated plasmid DNA of IL 4 as a transgene suppression of TNBS induced colitis[43,44], or it can be pro inflammatory with respect to allergic disorders[45].

IL-4 will impede the development of TH1-dominated disease and TNBS/DNBS is often considered in this category[46,47]. In this context, IL 4 was found to suppress LPS induced IL 12p40 production by DCs, an outcome enhanced in cells treated with HD antigen. Indeed, HD antigen evoked DC production of IL-10 when coupled with LPS exposure; an inability for DCs to produce IL 10 abrogated the capacity of the worm antigen to inhibit LPS-evoked IL-12p40 (unpublished observation). Although IL-4 is reported to drive pro-inflammatory responses in some contexts, we note that IL 4 only significantly exacerbated LPS induced IL 12 when added prior to LPS challenge; this was significantly attenuated by HD antigen. In addition, IL-4 and IL-10 are often functionally coupled, underscoring the finding that adoptive transfer of HD DCs invariably increased IL 4 and IL 10 output from the recipient's splenocytes. Similarly, transfer of DCs pulsed with schistosome egg antigen resulted in up-regulation of IL-4 and IL-10, with IL-10 synthesis being IL-4Rα-dependent[21]. In addition, the expansion of IL 10^+CD4^+ T cells in *Nippostrongylus brasiliensis* infected mice is significantly impaired in mice lacking the IL-4Rα, and this ultimately prevented the establishment of IL-4 responses[48]. Alternatively, IL-10 treated DCs induced IL-4 production in $CD4^+$ cells[24].

IL-4 promotes the production and secretion of mucin[21] that protects the gut from irritants in the lumen and this is likely a component of the anti colitic effects mediated by this cytokine. Finally, and directly linked to infection with helminth parasites and hence HD-DCs, IL-4 drives the development of alternatively activated macrophages (AAMs or M(IL-4)) that have been implicated in wound healing[48] and the suppression of colitis-induced by DNBS, oxazolone and dextran sodium-sulfate (DSS)[26,30,31].

Intriguingly, IL-4-treated DCs adopt an alternatively activated phenotype (AADC) reminiscent of the AAM, as characterized by increased expression of Ym1 and Relm α[31]. We confirmed these findings and also noted up regulation of arginase 1, which has been implicated in the suppression of colitis[52,53] and is important in tissue repair after injury[54,55]. Comparison with AAMs predicts an anti-colitic ability of AADCs; however, this was not observed when IL-4 evoked AADCs were compared directly with HD-DCs in the DNBS model of colitis. These data imply that HD antigen elicits a unique regulatory phenotype, a speculation supported by data showing that HD-DCs displayed no increases in Ym1 or Relm α mRNA and only small increases in arginase 1 mRNA, and HD antigen actually inhibited IL-4 induction of Relm-α mRNA in DCs. In accordance with these findings, a variety of helminth extracts have been shown to affect the behavior of DCs and modify their response to pathogen-associated molecular patterns (see above)[16,56,57].

Since transfer of HD-DCs evokes IL-4 production, and IL-4 as a paracrine or autocrine factor can direct DC function[57] (while noting that HD antigen did not evoke DC synthesis of IL-4), this raised the possibility that at least part of the role of IL-4 in mice given HD-DCs could be to influence the fate of the transferred DC. This would appear to be the case, as adoptive transfer of HD-DCs that lacked the IL-4Rα did not ameliorate DNBS-induced colitis to any significant degree. Given that lack of IL-4Rα does not affect the DCs ability to present antigen[57], we surmise that IL-4 signaling is of direct importance to the HD-DC regulatory program. The use of IL-4R$\alpha^{-/-}$ HD-DCs highlighted the reciprocity in the regulation of IL-4 and IL-10 production and the inhibition of colitis, since splenocytes from mice given these cells displayed no increase in IL-4 or IL-10. Interaction between parasite molecules and host factors in the regulation of host cell function is not unprecedented: for example, helminth-products and host antibodies cooperated to enhance macrophage-myofibroblast communication that mediated intestinal wound repair[58].

In summary, using the DNBS model of colitis that recapitulates some aspects of human inflammatory bowel disease, and considering cellular immunotherapy as a novel facet of helminth therapy for inflammatory disease, the data obtained support three conclusions: (1) HD-DCs, but not IL-4 educated AADCs, block DNBS-induced colitis; (2) the reduced severity of DNBS-induced colitis due to adoptive transfer of HD-DCs is dependent on IL-4α signaling in both the recipient mice and in the HD-DCs, indicating that the worm antigen synergizes with host IL-4 to elicit a regulatory program in the DCs; and, (3) cross-talk between IL-4 and IL-10 is required for the anti colitic effect of the HD-DCs (Fig. 8). Much remains to be done to define the effector mechanisms that underlie the HD-DC inhibition of colitis. However, the need for IL-4Rα-IL-4 signaling in the donor cells and in the recipients adds to awareness of the complexity of the *in vivo* events needed for the HD-DC to be effective, and that would likely be essential in patients identified as possible candidates for this novel therapeutic approach.

Methods

Animals. Animal experiments were performed in accordance with the Canadian Council on Animal Welfare, under a protocol approved by the University of Calgary Animal Care Committee (protocol AC13–0015). Balb/c mice (Charles River Laboratories), IL-10 KO mice (Jackson Laboratories), and IL-4R$\alpha^{-/-}$ mice and their WT controls (BALB/c background; gift from Dr. Frank Brombacher) were maintained at the Animal Resources Centre, University of Calgary, with free access to chow and water. Mice were used between 8–12 weeks of age.

HD antigen. Adult *H. diminuta* (HD) worms were recovered from the small intestine of infected rats, washed thoroughly, homogenized in PBS, and centrifuged (4000 rpm, 30 min, 4 °C). The PBS soluble fraction was removed and centrifuged a second time and protein content determined via Bradford Assay; aliquots of HD antigen were stored at −80 °C. Several batches of HD antigen were used throughout these experiments, all of which were standardized by their ability to suppress LPS-induced TNFα production by THP1 monocytic-like cells (48 hours, 1 μg/mL) by ≥40%[31]. A ToxinSensor Chromogenic LAL kit (Genscript) revealed trace levels (65 pg LPS/mg HD antigen) of endotoxin contamination[59].

HD-DC generation and adoptive transfer. Bone Marrow (BM) cells were harvested and seeded at 5×10^5/10 mL in RPMI medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% FBS, 2% Pen/Strep, 1% sodium pyruvate and Glutamax™ (all Gibco, Grand Island, NY), with recombinant murine GM-CSF (20 ng/mL; Peprotech) for differentiation to dendritic cells (DC). Cells were fed an additional 10 mL of supplemented medium and 20 ng/mL of GM-SCF on Day 3, and another 5 mL of medium with 40 ng/mL GM-SCF on day 6. On Day 8, floating and loosely adherent cells were removed by gentle pipetting. Cells were 91 ± 2% $CD11c^+$ as determined by flow cytometry. Cells were washed, resuspended in 10% DMSO in FBS ($5–10 \times 10^6$/mL) and stored in liquid nitrogen. For *in vitro* experiments, cells were treated with HD antigen (100 μg/mL), 24 h prior to exposure of LPS (1 μg/mL) ± IL-4 (20 ng/mL); supernatants were collected 24 h later. For adoptive transfer experiments, cells were left untreated, treated with IL-4 (20 ng/mL), with HD extract (100 μg/mL), or both for 24 hours; doses of IL-4 and HD were selected based on previously published methods for AADC generation, and immunomodulation of THP-1 cells, respectively[30,31,55]. Cells were washed (x3) with sterile PBS, and 1×10^6 cells in 500 μL of sterile PBS injected intraperitoneally (i.p.), 48 h prior to dinitrobenzene sulfonic acid (DNBS) challenge.

Induction of Colitis. Mice were anesthetized (Inhaled Isoflurane (4%)) and colitis induced by intra-rectal (i.r.) instillation of 3 mg DNBS (MP Biomedicals, Santa Barbara, CA) dissolved in 100 μl of 50% ethanol (PBS as negative control) using a polyethylene catheter inserted approximately 3 cm from the anus[7].

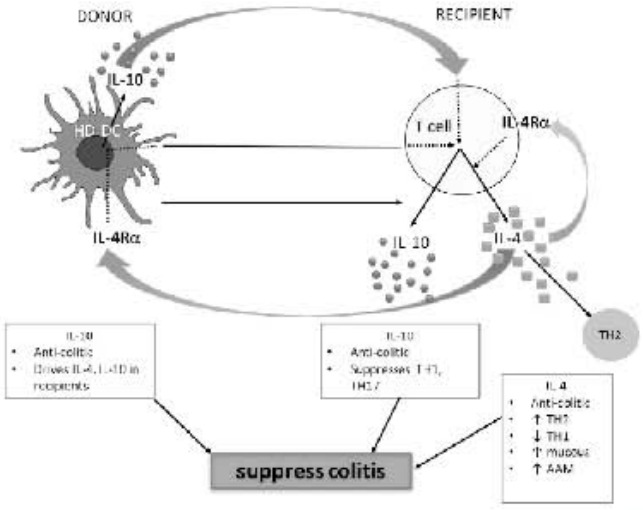

Figure 8. Summary model of the role of IL-4 signaling in HD-DC suppression of colitis. Recipient IL-4Rα signaling is required for HD-DC induction of IL-4, and suppression of colitis. HD-DCs induce IL-4 production in recipients, which likely enhances recipient IL-4 production via autocrine signaling through IL-4Rα. Activation of IL-4Rα is required to drive the regulatory anti-colitic phenotype of HD-DCs, in addition to splenic IL-4 and IL-10 in recipient mice. As HD-DCs are not a source of IL-4, HD-DC induced recipient IL-4 is the likely source for IL-4Rα activation on HD-DCs. HD-DCs produce IL-10 *in vivo*, and drive synthesis of IL-4 and IL-10 in recipient mice. Overall, IL-10 is an anti-colitic cytokine that can suppress TH1 and TH17 pro-inflammatory responses, and is required to establish Th2 immune responses; IL-4 is also anti-colitic, driving TH2 and suppressing TH1 immune responses, driving the alternative activation of macrophages, and enhancing mucus production.

Gene	Forward	Reverse
18s	GTAGTTGAACCCCATT	CGGTAGTAGCG
arg-1	GTCTGTTGGGGCGAAT	GATGCTTCCAACTGCCAGAGC
mrnlc	GGAACTTCTTGCCAATCCAGC	AAGCACATCCAGTAGCAGTC
chil-3	GTACCCTGGGTCTCCAGGAA	CCTTGGAATGTGCTTTCTCCACAG

Table 1. List of Primers used.

Assessment of Colitis. Mice were examined daily for signs of disease and weight change. Mice were euthanized 72 h after the induction of colitis, and the colon was removed for assessment and a macroscopic damage score was calculated as previously published: presence of visceral adhesions (0–2 points); colonic shortening (relative to controls, multiplied by 0.1), proportion of colonic inflammation (% inflamed regions multiplied by 0.05), diarrhea, fecal blood, and erythema (0–1 point each)[37]. Proportion of colon shortening and colon inflammation were multiplied by factors to produce a weighted value that represents approximately 20% of the total severity of macroscopic disease observed in the DNBS model of colitis, based on our previous observations of the positive control group (e.g. The average colonic shortening of DNBS treated animals is approximately 25%, so multiplying by 0.1 gives 2.5 points, or approximately 20% of the damage score, assuming an approximate damage score of 12 points). Weight change was not included in the macroscopic disease score, as our intention was to focus on colon pathology. A 1 cm portion of the colon, measured 20% from the distal end, was fixed in formalin, embedded in paraffin, sectioned (5 μm) and stained with haematoxylin and eosin (H&E). Histopathology was assessed in a blinded fashion using a 12 point scale: cellular infiltration (0–3 points); loss of architecture (0–3 points); muscle thickening (0–2 points); goblet cell depletion, crypt abscess, edema, and ulcers (0–1 point each)[9].

Real Time PCR. RNA from cells was isolated with TRizol reagent (Invitrogen) following the manufacturer's instructions, quantified with a Nanodrop Spectrophotometer, and used to generate cDNA with the iScript kit (Bio-Rad Laboratories, Mississauga, ON, Canada). PCR primers were created using the NCBI Primer BLAST program, and synthesized by Invitrogen. Primers used are listed in Table 1.

Flow Cytometry. Splenocytes (5×10^6/mL) were stimulated with plate-bound anti-CD3 antibodies (2 μg/mL) and soluble anti CD28 antibodies (2 μg/mL) for 3 days, and brefeldin A (5 μg/mL; Biolegend) for the final 16 h of culture. Cells were resuspended in flow buffer (PBS with 0.1% sodium azide and 1% FBS), and stained with anti-CD16/32 TruStain fcX (1: 200, Biolegend) and a live/dead viability dye (BV-510 at 1:500; BD Biosciences) for 30 min at 4 °C. Staining of surface markers (APC-CD4, FITC-CD8α, PercpCy5.5-CD19, all Biolegend) was performed for 30 min at 4 °C in the dark. Cells were fixed, permeabilized using the FIX/PERM set (Biolegend) and blocked in 5% rat serum for 10 min at room temperature prior to intracellular staining for IL-4 with PE Cy7 anti IL-4 (Biolegend) for 20 min at room temperature. Data was analyzed using BD FACSDIVA software (BD Biosciences, San Jose, CA, USA).

Western Blotting. Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris HCL pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS, and protein content measured. Protein samples (40 μg) were boiled with loading dye, loaded on a 12% SDS-PAGE gel, transferred to a nitrocellulose membrane, and blocked with 5% nonfat milk in PBS. Samples were probed for β-actin (anti-goat, Santa Cruz Biotechnology, Santa Cruz, CA), arginase 1 (anti-rabbit, Santa Cruz), and Ym-1 (anti-rabbit, Stem Cell Technologies, Vancouver BC, Canada) at concentrations of 1:1000, 1:200, and 1:500 respectively. Following incubation with horseradish peroxidase conjugated secondary antibodies (1:1000–2000), membranes were washed in tris-buffered saline with 1% tween-20, stripped using 100 mM β-mercaptoethanol buffer for 10 min at 50 °C. Blots were developed and densitometry accomplished using Gel Capture MicroChemi Chemiluminescence Substrate.

Cytokine analysis. Cytokine levels were assessed in the supernatants of a) BMDCs left untreated or treated with HD antigens (100 μg/mL, 24 h) and b) splenocytes (5×10^6) stimulated with concanavalin A (2 μg/mL) for 48 h. ELISAs were performed using DuoSet® kits from R&D Systems (Minneapolis, MN), following the manufacturers' instructions.

Statistical Analysis. All data are displayed as mean ± SEM; $p < 0.05$ was accepted as statistically significant. A D'Agostino Pearson omnibus normality test was used to assess a Gaussian distribution. For parametric data, statistical outliers were removed using the Grubb's test, and a one-way ANOVA with Tukey's post-test, or Sidak's multiple comparison test was performed to assess significance between groups. For repeated measure ANOVAs, a Dunnett's multiple comparison post test was performed. When comparisons between two groups were being made, a two-tailed Student's t-test was used, and paired where appropriate; for comparison to a mean with a known value, a one-sided, two-tailed t-test was performed. Significant differences were assessed for non-parametric data using a Mann-Whitney or Kruskal-Wallis test with Dunn's post-test. A Spearman rank correlation was used to assess correlations between non parametric data. For macroscopic and histology damage scores, analysis were conducted on all DNBS treated groups; all other analysis included the negative control. Analyses were conducted with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

References

1. Strachan, D. P. Hay fever, hygiene, and household size. *Brit Med J* 299, 1259–1260 (1989).
2. Zaccone, P., Fehervari, Z., Phillips, J. M., Dunne, D. W. & Cooke, A. Parasitic worms and inflammatory diseases. *Parasite Immunol* 10, 515–523 (2006).
3. Helmby, H. Human helminth therapy to treat inflammatory disorders - where do we stand. *BMC Immunol* 16, 1–12 (2015).
4. Maizels, R. M. Parasitic helminth infections and the control of human allergy and autoimmune disorders. *Clin Microbiol Infect* 22, 481–486 (2016).
5. Wang, L. *et al. Heligmosomoides polygyrus bakeri* infection activates colonic Foxp3$^+$ T cells enhancing their capacity to prevent colitis. *J Immunol* 191, 1927–1934 (2013).
6. Blum, A. M. *et al. Heligmosomoides polygyrus bakeri* induces tolerogenic dendritic cells that block colitis and prevent antigen-specific gut T cell responses. *J Immunol* 189, 2512–2520 (2012).
7. Hunter, M. M., Wang, A., Hirota, C. L. & McKay, D. M. Neutralizing anti-IL-10 antibody blocks the protective effect of tapeworm infection in a murine model of chemically induced arthritis. *J Immunol* 174, 7368–7375 (2005).
8. Summers, R. W., Elliott, D. E. & Urban, J. F. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 128, 825–832 (2005).
9. Motomura, Y. *et al.* Helminth antigen-based strategy to ameliorate inflammation in an experimental model of colitis. *Clin Exp Immunol* 155, 88–95 (2009).
10. Correale, J. & Farez, M. Helminth antigens modulate immune responses in cells from multiple sclerosis patients through TLR2-dependent mechanisms. *J Immunol* 183, 5999–6012 (2009).
11. Carranza, F. *et al.* Helminth antigens enable CpG-activated dendritic cells to inhibit the symptoms of collagen-induced arthritis through Foxp3$^+$ regulatory T cells. *PLoS One* 7, e40356 (2012).
12. Sofronic-Milosavljevic, L. J. *et al.* Application of dendritic cells stimulated with *Trichinella spiralis* excretory–secretory antigens alleviates experimental autoimmune encephalomyelitis. *Med Microbiol Immunol* 202, 239–249 (2013).
13. Hsu, F. J. *et al.* Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 2, 52–58 (1996).
14. Morse, M. A. *et al.* A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. *Ann Surg* 258, 879–886 (2013).
15. Garcia, I. *et al.* A therapeutic dendritic cell-based vaccine for HIV-1 infection. *J Infect Dis* 203, 473–478 (2011).
16. Hasselblatt, P. *et al.* Remission of refractory Crohn's disease by high-dose cyclophosphamide and autologous peripheral blood stem cell transplantation. *Aliment Pharmacol Ther* 36, 735–735 (2012).
17. Forbes, G. M. *et al.* A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* 12, 64–71 (2014).
18. Cicchiello, R. *et al.* Autologous bone marrow–derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut* 60, 788–798 (2011).
19. de la Portilla, F. *et al.* Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis* 28, 313–323 (2013).
20. Metwali, A. E. *et al.* Adoptive transfer of helminth antigen-pulsed dendritic cells protects against the development of experimental colitis in mice. *Eur J Immunol* 45, 3126–3139 (2015).

21. Cook, P. C. *et al.* Alternatively activated dendritic cells regulate CD4+ T-cell polarization *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* **109**, 9977–9982 (2012).
22. Gordon, S. Alternative activation of macrophages. *Nat Rev Immunol* **3**, 23–35 (2003).
23. Marecol, A. *et al.* Interleukin-4 can induce interleukin-4 production in dendritic cells. *Immunol* **117**, 271–279 (2006).
24. Liu, L., Rich, B. E., Inobe, J., Chen, W. & Weiner, H. L. Induction of Th2 cell differentiation in the primary immune response: dendritic cells isolated from adherent cell culture treated with IL-10 prime naive CD4+ T cells to secrete IL-4. *Int Immunol* **10**, 1017–1026 (1998).
25. Boks, M. A. *et al.* IL-10-generated tolerogenic dendritic cells are optimal for functional regulatory T cell induction-a comparative study of human clinical applicable DC. *Clin Immunol* **142**, 332–342 (2012).
26. Leung, G. *et al.* Cryopreserved IL-4-treated macrophages attenuate murine colitis in an integrin β7-dependent manner. *Mol Med* **21**, 934–936 (2015).
27. Yao, Y., Li, W., Kaplan, M. H. & Chang, C. H. Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. *J Exp Med* **201**, 1899–1903 (2005).
28. Keely, S. *et al.* Contribution of epithelial innate immunity to systemic protection afforded by prolyl hydroxylase inhibition in murine colitis. *Mucosal Immunol* **7**, 114–123 (2014).
29. Anthony, R. M., Rutitzky, L. I., Urban, J. F. (Jr.), Stadecker, M. J. & Gause, W. C. Protective immune mechanisms in helminth infection. *Nat Rev Immunol* **7**, 975–987 (2007).
30. Lopes, F. *et al.* Helminth regulation of immunity: a three pronged approach in treating colitis. *Inflamm Bowel Dis* **10**, 2459–2472 (2016).
31. Allen, J. E. & Maizels, R. M. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol* **11**, 375–388 (2011).
32. Ma, Y. L. *et al.* IL-4-producing dendritic cells induced during *Schistosoma japonica* infection promote Th2 cells via IL-4-dependent pathway. *J Immunol* **195**, 3769–3780 (2015).
33. van Panhuys, N. *et al.* Basophils are the major producers of IL-4 during primary helminth infection. *J Immunol* **186**, 2719–2728 (2011).
34. Connor, L. M., Tang, S. C., Camberis, M., Le Gros, G. & Ronchese, F. Helminth-conditioned dendritic cells prime CD4+ T cells to IL-4 production *in vivo*. *J Immunol* **193**, 2708–2717 (2014).
35. Dowling, D. J. *et al.* *Ascaris lumbricoides* pseudocoelomic body fluid induces a partially activated dendritic cell phenotype with Th2 promoting ability *in vitro*. *Int J Parasitol* **41**, 253–261 (2011).
36. Falcon, C. *et al.* Excretory-secretory products (ESP) from *Fasciola hepatica* induce tolerogenic properties in myeloid dendritic cells. *Vet Immunol Immunopathol* **157**, 46–48 (2014).
37. McKay, D. M. & Khan, W. I. STAT-6 is an absolute requirement for murine rejection of *Hymenolepis diminuta*. *J Parasitol* **89**, 188–189 (2003).
38. Hunter, M. M., Wang, A. & McKay, D. M. Helminth infection enhances disease in a murine TH2 model of colitis. *Gastroenterology* **132**, 1320–1330 (2007).
39. Reyes, J. L. *et al.* Splenic B cells from *Hymenolepis diminuta* infected mice ameliorate colitis independent of T cells and via cooperation with macrophages. *J Immunol* **194**, 364–378 (2015).
40. Harris, D. P., Goodrich, S., Mohrs, K., Mohrs, M. & Lund, F. E. The development of IL-4 producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor, and Th2 cells. *J Immunol* **175**, 7103–7107 (2005).
41. Noben-Trauth, N., Hu, Y., Li, J. & Paul, W. E. IL-4 secretion from individual naive CD4+ T cells acts in an autocrine manner to induce Th2 differentiation. *Eur J Immunol* **32**, 1428–1433 (2002).
42. Liu, Z., Liu, Q., Hamad, H., Anthony, R. M., Foster, A., Finkelman, F. D., Urban (Jr.), J. F. & Gause, W. C. IL-3 and exocrine IL-4 drive the *in vivo* development of antigen specific Th2 T cells elicited by nematode parasites. *J Immunol* **174**, 2242–2249 (2005).
43. Hogaboam, C. M. *et al.* Therapeutic effects of interleukin-4 gene transfer in experimental inflammatory bowel disease. *J Clin Invest* **100**, 2766–2776 (1997).
44. Xiong, J. *et al.* Effects of interleukin-4 or interleukin-10 gene therapy on trinitrobenzenesulfonic acid-induced murine colitis. *BMC Gastroenterol* **13**, 161–171 (2013).
45. Gandhi, N. A. *et al.* Targeting key proximal drivers of type 2 inflammation in disease. *Nat Rev Drug Discov* **15**, 35–50 (2016).
46. Neurath, M. F., Finotto, S. & Glimcher, L. H. The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* **8**, 567–573 (2002).
47. Abbas, A. K., Murphy, K. M. & Sher, A. Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793 (1996).
48. Baltz, A., Harcus, Y. M., Taylor, M. D., Brombacher, F. & Maizels, R. M. IL-4R signaling is required to induce IL-10 for the establishment of Th2/2 immunity. *Int Immunol* **18**, 1421–1431 (2006).
49. Jung, J. C. & Nair, M. G. Alternatively activated macrophages revisited: new insights into the regulation of immunity, inflammation and metabolic function following parasitic infection. *Curr Immunol Rev* **9**, 147–156 (2013).
50. Leung, G., Wang, A., Fernando, M., Phan, V. C. & McKay, D. M. Bone marrow-derived alternatively activated macrophages reduce colitis without promoting fibrosis: participation of IL-10. *Am J Physiol Gastrointest Liver Physiol* **304**, G281–G292 (2013).
51. Hunter, M. M. *et al.* *In vitro*-derived alternatively activated macrophages reduce colonic inflammation in mice. *Gastroenterology* **138**, 1395–1405 (2010).
52. Gobert, A. P. *et al.* Protective role of arginase in a mouse model of colitis. *J Immunol* **173**, 2109–2117 (2004).
53. Weisser, S. B. *et al.* Arginase activity in alternatively activated macrophages protects PI3Kp110δ deficient mice from dextran sodium sulfate induced intestinal inflammation. *Eur J Immunol* **44**, 3593–3607 (2014).
54. Campbell, L., Saville, C. R., P. J., M., Cruickshank, S. M. & Hardman, M. J. Local arginase 1 activity is required for cutaneous wound healing. *J Invest Dermatol* **133**, 2461–2470 (2013).
55. Dzik, J. M. Evolutionary roots of arginase expression and regulation. *Front Immunol* **5**, 1–11 (2014).
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. *Mol Immunol* **44**, 2605–2615 (2007).
57. Goodridge, H. S. *et al.* Immunomodulation via novel use of TLR4 by the filarial nematode phosphorylcholine-containing secreted product, ES-62. *J Immunol* **174**, 284–293 (2005).
58. Esser-von Bieren, J. *et al.* Immune antibodies and helminth products drive CXCR2-dependent macrophage-myofibroblast crosstalk to promote intestinal repair. *PLoS Pathog* **11**, e1004778 (2015).
59. Johnston, M. J. *et al.* Extracts of the rat tapeworm, *Hymenolepis diminuta*, suppress macrophage activation *in vitro* and alleviate chemically induced colitis in mice. *Infect Immun* **78**, 1904–1915 (2010).

Acknowledgements

Funding for this study was provided by Natural Sciences and Engineering Research Council of Canada (NSERC) grant to D.M. McKay. C.F. Matisz held Alberta Innovates-Health Solutions (AI-HS) and Canadian Institutes for Health Research (CIHR) PhD studentships. E. Thompson was supported by the National Sciences and Engineering Research Council (NSERC) CREATE Host Parasite Interactions program of the University of Calgary. F. Lopes is a recipient of CIHR-Canadian Association of Gastroenterology (CAG)/Allergan Inc., and AI-HS post-doctoral fellowships. A. Al Rajabi was recipient of an Eyes High post-doctoral fellowship from the Univ. Calgary. D.M. McKay holds a Canada Research Chair (Tier 1) in Intestinal Immunophysiology in Health

and Disease, and K.A. Sharkey holds a Crohn's and Colitis Canada Chair in IBD Research at the University of Calgary. We thank Dr. Karen Poon, manager of the Nicole Perkins Microbial Communities Core Labs, for her assistance with Flow Cytometry.

Author Contributions

C.M., D.M. and K.S. drafted and made critical revision of the articles, and alongside F.L. and L.T. contributed to experimental conception and design. Data collection, analysis, and interpretation was done by C.M., B.F.-L., F.T. F.L., A.A.R. and A.W. All authors reviewed the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Matisz, C. E. *et al.* Suppression of colitis by adoptive transfer of helminth antigen-treated dendritic cells requires interleukin-4 receptor α signaling. *Sci. Rep.* **7**, 40631; doi: 10.1038/srep40631 (2017).

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 8634603, 11 pages
http://dx.doi.org/10.1155/2016/8634603

Review Article

Role of Macrophages in the Repair Process during the Tissue Migrating and Resident Helminth Infections

Berenice Faz-López,[1] Jorge Morales-Montor,[2] and Luis I. Terrazas[1,3]

[1]*Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), 54090 Tlalnepantla, MEX, Mexico*
[2]*Departamento de Inmunología, Instituto de Investigaciones Biomédicas, UNAM, 04510 Ciudad de México, Mexico*
[3]*Laboratorio Nacional en Salud, Facultad de Estudios Superiores Iztacala, UNAM, 54090 Tlalnepantla, MEX, Mexico*

Correspondence should be addressed to Luis I. Terrazas; literrazas@campus.iztacala.unam.mx

Received 15 February 2016; Revised 13 May 2016; Accepted 19 July 2016

Academic Editor: Gernot Zissel

The Th1/Th2/Th17 balance is a fundamental feature in the regulation of the inflammatory microenvironment during helminth infections, and an imbalance in this paradigm greatly contributes to inflammatory disorders. In some cases of helminthiasis, an initial Th1 response could occur during the early phases of infection (acute), followed by a Th2 response that prevails in chronic infections. During the late phase of infection, alternatively activated macrophages (AAMs) are important to counteract the inflammation caused by the Th1/Th17 response and larval migration, limiting damage and repairing the tissue affected. Macrophages are the archetype of phagocytic cells, with the primary role of pathogen destruction and antigen presentation. Nevertheless, other subtypes of macrophages have been described with important roles in tissue repair and immune regulation. These types of macrophages challenge the classical view of macrophages activated by an inflammatory response. The role of these subtypes of macrophages during helminthiasis is a controversial topic in immunoparasitology. Here, we analyze some of the studies regarding the role of AAMs in tissue repair during the tissue migration of helminths.

1. Introduction

Helminth infections are a worldwide public health and economic problem due to their high morbidity rather than mortality. These infections are associated with socioeconomic (poor hygiene), demographic (living in endemic zones), health (obesity, diabetes, and viral infections such as human immunodeficiency virus (HIV)), and biological (raw meat consumption, sex, age, and immune response) factors, among others [1].

The clinical manifestations are diverse and include self-limited diarrhea, respiratory symptoms such as cough, wasting syndrome, and anemia. In severe infections, some people develop asthma-like symptoms [2] and neurologic disorders when the pathogen has the ability to migrate into the brain, such as in neurocysticercosis by *Taenia solium* [3, 4] and *Toxocara canis* infection [5], or motor disorders such as those occurring in *Trichinella spiralis* [6] and *T. canis* infections [7]. The diversity of symptoms caused by helminths is related to the organs they migrate to during their life cycle, such as the lung (*Ancylostoma duodenale*, *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Brugia malayi*, *Dirofilaria immitis*, *T. canis*, *Schistosoma mansoni*, *Echinococcus granulosus*, and *Nippostrongylus brasiliensis*) [2], gall bladder and liver (*Schistosoma* sp., *Toxocara* sp., and *A. lumbricoides*), muscle (*T. canis*, *T. spiralis*) [6, 7], and brain (*T. canis*, *Taenia* sp.) [3, 4].

In contrast to the prevailing consensus, many helminth infections are not permanent residents of the bowels; instead, they have the ability to migrate through different organs or persist at a location for weeks, months, or years. During their migration, helminths secrete proteolytic enzymes, such as serine proteases and cysteine proteases, with fibrinolytic

effects that cause the disruption of cell junctions and the extracellular matrix, enabling entry to different organs [8–10] and causing tissue damage along the path of the migration.

2. Tissue Damage and Repair or Healing

When the larvae of helminths migrate through the host's body and destroy cells and tissues in their path, these cells and damaged tissue must be rapidly repaired. To achieve this, the host must turn on a series of coordinated biochemical and cellular events focused on the replacement of the destroyed tissue with living and functional tissue. Such a process is called tissue repair or tissue healing [11] and includes at least 4 events: bleeding, inflammation, proliferation, and remodeling (Figure 1) [12]. Bleeding and inflammation are early events that occur over hours or a few days, whereas proliferation and remodeling take weeks or even months. Vasculature changes such as vasodilatation and vasopermeability are rapidly induced to recruit cells to the site of damage, where prostaglandins and serotonin play an essential role. Once sufficient cell populations arrive at the site of the damaged tissue, the inflammatory stage ensues, with mast cells, neutrophils, and platelets as well as inflammatory $Ly6C^{hi}$ monocytes and resident macrophages playing an important role in containing bleeding and initializing the first steps of bleeding control. Some of these cells (neutrophils and macrophages) have strong phagocytic activity and help to remove debris in the wound, an essential step in the repair process. This stage is mainly driven by macrophages and fibroblasts, which become activated and initiate the production or expression of different molecules such as platelet-derived growth factor (PDGF). PDGF induces fibroblast proliferation and promotes fibrogenesis, collagen precursors, and integrins that help in the communication among the extracellular matrix, inflammatory cells, fibroblasts, and parenchymal cells [12, 13]. Here, macrophages play a critical role as providers of many of the molecules necessary for tissue repair, including arginase-1, transforming growth factor- (TGF-) β, FIZZ-1 (found in inflammatory zone), and fibrin, which are involved in fibrosis by inducing myofibroblast differentiation, key elements in collagen (mainly type III collagen) and fibrin deposition [11]. Another cell population recently implicated with the repair processes in the lungs and intestines are the type 2 innate lymphoid cells (ILC2s), which are a source of cytokines and other components that participate in the early steps of the healing process [15]. This inflammatory stage must be mainly acute (1–4 days) to avoid excessive collagen accumulation and fibrosis, elements that may alter tissue architecture. Thus, these early events prepare the tissue for the next step in healing-proliferation. The proliferation of fibroblasts is a key event in this stage because they will migrate to the damaged area from similar neighboring tissue. Here, macrophages again play a primary role as providers of macrophage-derived growth factors (including fibroblast growth factor) that activate and induce proliferation in fibroblasts to provide new cells to repopulate the affected tissue [16]. The final and longest step in the repair and healing of the tissue consists of remodeling, which involves the reorganization of collagen fibers, which are "weak or fine" fibers occurring during the inflammation and proliferation stages. However, in the remodeling step, these fibers become strong because collagen type III is replaced with collagen type I, with more cross-links, as it enters into a reorientation process [14]. Thus, collagen synthesis continues during this final step to "mimic" the damaged tissue as much as possible indicating that tissue repair is a dynamic and long-lasting process.

3. Immune Activation by Helminths

In addition to proteolytic enzymes, helminths generate immunomodulatory antigens that induce predominantly Th2-biased responses. This type of immunity is characterized by the production of different immunoglobulin (Ig) subclasses, such as IgE, IgG1, and IgG4, as well as interleukins, IL-4, IL-13, IL-5, and IL-10, which benefit the expansion of diverse cellular subpopulations such as eosinophils, mast cells, helper T cells, and alternatively activated macrophages (AAMs). The interactions among these different cell types and antibodies promote allergy and hypersensitivity, which are related to the increase in vascular permeability, angiogenesis, cellular recruitment, smooth muscle contraction, mucus secretion by goblet cells, and collagen deposits, which together are important mechanisms of defense against invasive helminth infections [17].

Another cell population associated with these parasites is the $CD4^+Foxp3^+$ regulatory T cells (Tregs), which are essential to maintain immune homeostasis and prevent autoimmunity; however, they are also involved in the control of T_H1 and T_H2 immune responses in some infectious diseases. In fact, several reports have confirmed that an increase in Treg numbers is associated with different helminth infections, where they play a dual role. Such an increase in the regulatory cells appears to be permissive for parasites, whereas these high numbers of helminth-induced $Foxp3^+$ Tregs dampen potentially pathogenic inflammatory responses or exacerbate Th2 responses in the tissue where the helminth has been allocated. Such conclusions were obtained by removing, stimulating, or transferring $Foxp3^+$ Tregs using different experimental designs [18].

More recently, other cell populations were related to helminth infections, ILC2s. This cell population comprises a limited number of cells but appears to play an important role in both protective and repair processes in mucosal tissues. They are classified into two different populations: natural ILC2s (nILC2s) and inflammatory ILC2s (iILC2s). ILC2s have been found in the steady state in many organs, such as the lungs, liver, spleen, intestinal lamina propria, skin, bone marrow, and adipose tissue. These cells are stimulated by thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 and after helminth infection produce effector cytokines, such as IL-4 and IL-13, and provide an important source of Th2-type cytokines. Thus, this process triggers the cell recruitment of eosinophils and macrophages, increasing the production of IL-13, IL-5, and IL-9, mucus production by goblet cells, muscle contraction, mastocytosis, tissue repair, and metabolic homeostasis [19]. The repair process by ILC2s is mostly associated with the early activation of AAMs, which can trigger the repair process via arginase-1 (Arg-1).

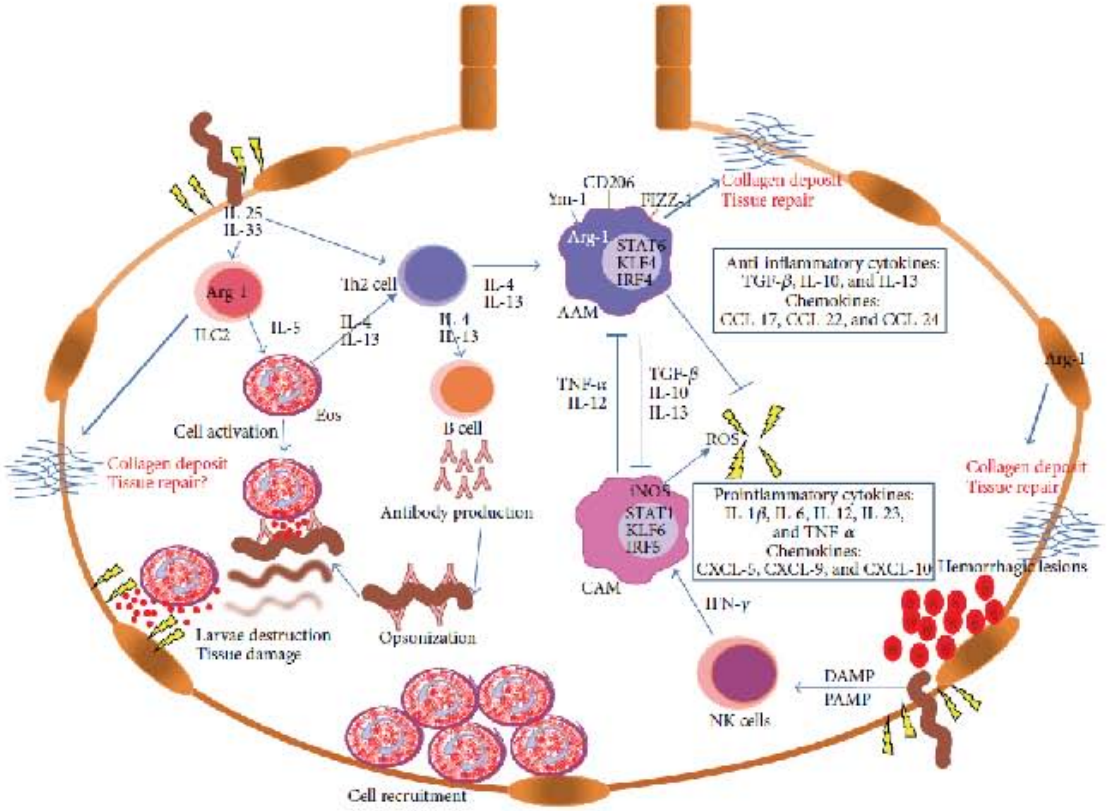

Figure 1: Immune response in tissue helminth infection. Lung tissue is usually affected during helminth migration; therefore, many repair mechanisms have been described for pulmonary tissue. The immune response is triggered when helminths disrupt the epithelial barrier. Helminths are a source of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs), which activate various cells such as NK cells, epithelial cells, and innate lymphoid cells (ILCs). The production of IL-25 and IL-33 also activates ILCs, which are a main source of IL-5 that is important in eosinophil (Eos) activation. Eos bind antibodies linked to the parasite surface and release their intracytoplasmic enzymes during the acute phase of the infection, allowing parasite elimination; however, the surrounding tissue is also damaged. IL-25 and IL-33 also activate T helper lymphocytes type 2 (Th2), which in turn secretes IL-4 and IL-13, which promote B cell activation, antibody production, and the induction of alternative activated macrophages (AAMs). AAMs have two important mechanisms to decrease tissue damage. First, they inhibit the cytotoxic effect produced by classically activated macrophages (CAMs). Second, they produce enzymes, such as arginase 1 (Arg 1), that promote collagen production and deposition on damaged tissue, therefore restoring the function lost during CAMs and parasite-induced injury. AAMs also produce various cytokines (IL 10 and TGF β) and chemokines (CCL 17, CCL 22, and CCL 24) and express markers such as YM-1, FIZZ-1, and MMR. On the other hand, CAMs are activated through IFN-γ production by natural killer (NK) cells and produce proinflammatory cytokines (IL 1β, IL 6, IL 12, IL 23, and TNF α) and chemokines (CXCL 5, CXCL 9, and CXCL 10) and express iNOS that produces reactive oxygen species (ROS) and causes tissue damage. It is important to notice that although AAMs are fundamental in tissue repair, other cells such as ILCs and epithelial cells, which constitutively express Arg 1, may aid in tissue repair and produce collagen deposits in the damaged tissue.

However, a recent report has indicated that ILC2s can produce this enzyme both under basal conditions and in response to helminth infection; thus, ILC2s have the potential to participate in tissue repair [20].

During helminth infections, cytokines are essential to activate various cell types, including eosinophils, which are involved in the mediation of most helminth infections [21]. Eosinophils are mainly activated by IL-5 and are an important source of IL-4 and IL-13, which enable the activation of Th cells, AAMs, and mast cells. In addition to cytokine production and cell activation, eosinophils can neutralize and eliminate tissue parasites through the secretion of granules that contain proteins, such as eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil derived neurotoxin (EDN), and eosinophil cationic protein (ECP) (Figure 1) [22].

These parasite killing mechanisms have been described in multiple studies. For example, Masure et al. measured the survival of *Ascaris suum* second-stage larvae in the presence of eosinophils, and they observed an important reduction in larvae survival associated with the degranulation

of eosinophils. The authors concluded that eosinophils are important immune cells in the defense against *A. suum* invasive larvae [23]. Other experimental models confirmed the protective role of eosinophils against multiple helminths, including *Strongyloides stercoralis* [24], *N. brasiliensis* [25], and *Heligmosomoides polygyrus* [26]. Nevertheless, during *T. canis* and *T. spiralis* infection, eosinophils do not seem to have such a protective role. For instance, in an *in vitro* model, Rockey et al. observed that eosinophils could attach to *T. canis* larvae and secrete granules; however, the larvae could separate from their sheaths and move away from eosinophils [4]. In another study, Takamoto et al. did not find a difference in the larvae burden of IL-5 deficient mice (characterized by having 3-fold lower circulating numbers of eosinophils), although eosinophils in WT mice were increased tenfold in the bone marrow and twenty-seven-fold in peripheral blood, and concluded that eosinophils do not play an important role in the clearance of *T. canis* larvae [27]. Another study using *T. spiralis* reported similar findings [28], suggesting that eosinophils do not enhance protective immunity also against this nematode.

Although it is clear that eosinophils play an important role in the protection against most helminths through the secretion of effector proteins, paradoxically, the proteins they release are sometimes harmful to the surrounding host tissues [29–32]. Evidence of this side effect was demonstrated in a model of *T. canis* infection, in which BALB/c mice were transfected with a plasmid encoding the IL-12 gene (pcDNA-IL-12) that inhibits the recruitment of eosinophils. The authors showed that transfected mice displayed reduced airway inflammation associated with a reduced eosinophilic infiltrate in the lungs and an increase in the Th1-type immune response characterized by elevated amounts of IL-12 and interferon-γ (IFN-γ) in this tissue [33]. In line with this idea, recently, a double-edged sword effect of eosinophils was demonstrated in experimental neurocysticercosis caused by *Mesocestoides corti*. Here, the authors used eosinophil-deficient mice to show that eosinophils are important cells for reducing the parasite load in the brain; however, these cells also intensify tissue damage and consequently worsen the disease outcome with more severe pathology [34].

Thus, after the damage to the host caused by helminth migration and immune cell activation, it is imperative that the tissue repair type 2 immune response plays a direct role in wound healing through the production of mediators that directly enhance the tissue repair process and through the control of inflammation, in which AAMs appear to have a central role [35]. However, a type 2 immune response takes several days; thus, a rapid mechanism for tissue repair is imperative early during the infection by helminths, when AAMs and their products appear to be crucial. In this regard, innate immune cells such as ILC2s may participate as an early source for IL-4 and IL-13 to rapidly induce AAMs.

4. Repair and Damage Mechanisms of Macrophages

Macrophage polarization to AAMs is related to the Th2 immune response and associated AAM cytokines, such as IL-4 and IL-13. Furthermore, diverse transcription factors, such as PU.1, signal transducer and activator of transcription 6 (STAT6), Kruppel-like factor (KLF) 4, and interferon regulatory factor (IRF) 4, are related to this type of macrophage [36]. Particularly, AAMs can be distinguished by their expression of diverse molecular markers, including the enzyme arginase-1 (Arg-1), members of the chitinase family (YM-1, YM-2, and AMCase), resistin-type molecules (FIZZ-1/Retnla/Relm-α, FIZZ-2/Retnlb/Relm-β, FIZZ-3/Retn/resistin, and FIZZ-4/Retnlg/Relm-γ), and TGF-β and mannose receptor (MMR/CD206) [37].

Moreover, classically activated macrophages (CAMs) are induced by Th1 immune responses, wherein IFN-γ plays a crucial role, and the transcription factors STAT1, KLF6, and IRF5 are implicated in their activation [36]. In contrast to AAMs, CAMs have enhanced antimicrobial actions mediated by the secretion of molecules such as nitric oxide (NO) and reactive oxygen species (ROS) that are essential for the destruction of intracellular pathogens (bacteria, viruses, and protozoan parasites). Additionally, CAMs are characterized by the production and secretion of proinflammatory cytokines, such as tumor necrosis factor-(TNF-)α, IL-12, and IL-1β [38]. Another difference between AAMs and CAMs is the expression by AAMs of Arg-1, which functions to metabolize L-arginine into L-ornithine. By contrast, CAMs use L-arginine to synthesize L-citrulline through inducible nitric oxide synthase (iNOS), producing NO and ROS, both mediators of their cytotoxic activity against intracellular pathogens and tissue damage [38].

5. Alternatively Activated Macrophage Functions

As previously mentioned, if macrophages are stimulated by IFN-γ, L-arginine is metabolized by iNOS, and the main cytokine induced is TNF-α or IL-12, macrophages will be classically activated. However, if there is predominance of Th2 cytokines, such as IL-4 and IL-13, there will be more AAMs that express Arg-1. Hence, AAMs expressing Arg-1 produce L-ornithine that can be metabolized into L-proline through ornithine aminotransferase (OAT), and L-proline is essential for collagen synthesis and tissue repair and regeneration [39]. AAMs also produce other elements involved in tissue repair, such as TGF-β and PDGF, which induce fibroblast proliferation and promote fibrogenesis and collagen production [40].

Another protein yielded by AAMs is YM-1, a member of the family of mammalian proteins that share homology to chitinases, which can bind chitin without chitinase activity. This protein has been associated with cellular recruitment and extracellular matrix deposition during tissue repair. Furthermore, FIZZ-1 is secreted in high amounts during inflammation, and it has been observed that diverse cells express this protein, including pneumocytes, alveolar epithelial cells, and macrophages. FIZZ-1 is also involved in fibrosis by inducing myofibroblast differentiation, key element in collagen and fibrin deposits [37, 41, 42]. Another immune factor related to AAM activation is antibody production. It has been described that some subclasses of antibodies

can reprogram macrophage gene expression and induce the production of repair-related molecules [43].

6. Alternatively Activated Macrophages in the Repair Process in Diverse Pathologies

The presence of AAMs in the repair process has been extensively studied in various pathologies where they play an active role and could be beneficial or harmful depending on the pathology. In a collagenase-induced intracerebral hemorrhage (ICH) mouse model, an increasing number of CX3CR1 macrophages were revealed to have AAM markers. When these macrophages were depleted, an increase in the ICH lesion volume was observed, and neurological deficits were more severe compared to those of control mice, indicating a protective role of these macrophages in ICH. From such data, the authors concluded that brain-infiltrating macrophages after ICH are polarized to the AAM phenotype, thereby contributing to recovery from such injury [44].

In asthmatic airway inflammation, the presence of AAM activated via PU.1 has a harmful effect on promoting the pathological progress of asthmatic airway inflammation. Such an effect was measured in conditional PU.1-deficient ($PU/ER(T)^{+/-}$) mice in response to the challenge of DRA (dust mite, ragweed, and *Aspergillus*) allergens, displaying attenuated allergic airway inflammation, decreased alveolar eosinophil infiltration, and reduced IgE production, changes associated with decreased mucus glands and goblet cell hyperplasia. To prove that AAMs were involved in the asthmatic airway pathology, macrophages from wild-type (WT) mice were differentiated with IL-4 and transferred to $PU/ER(T)^{+/-}$ mice showing an increase in asthmatic airway inflammation. When the mice were treated with tamoxifen to rescue PU.1 function, the pathology was worsened compared with that in mice transferred with macrophages. The data indicated that PU.1 plays a critical role in AAM polarization and, indeed, in the exacerbation of asthmatic inflammation pathology [45].

Other pathologies such as obesity and resistance to insulin are closely associated with inflammation. Obesity causes increased classical and decreased alternative macrophage activation, which in turn induces insulin resistance in target organs, as observed in a study where A_{2B} adenosine receptor (AR) activation results in important regulators of macrophage activation. A_{2B} AR deletion results in impaired glucose and lipid metabolism associated with increased inflammatory classical macrophage activation and inhibition of anti-inflammatory alternative macrophage activation. The expression of AAM transcription factors was also decreased in the adipose tissue of A_{2B} ARs-deficient mice, indicating that AAMs may play an important role in obesity and insulin resistance, and therapeutic strategies targeting A_{2B} ARs could be a preventive therapy for those pathologies [46].

In a recent report, the role for AAMs has also been highlighted in the reparative processes after myocardial infarction in adult mice, where higher numbers of AAMs were recruited to the infarcted area; such mechanism was IL-4 dependent [47].

In general, in pathologies different from those generated by tissue migrating or resident helminths, the presence of AAMs in the repair process is beneficial to counteract the effects of CAMs associated with a Th1 inflammatory immune response, with the exception of asthma where their presence seems to be more harmful than beneficial.

7. Role of Macrophages during Tissue Migrating and Resident Helminths

7.1. Nippostrongylus brasiliensis. This nematode, like many others, has a tissue migration phase in which it migrates throughout the lungs, causing alveolar hemorrhage and inflammation. The role of the immune response during acute lung injury caused by *N. brasiliensis* has been studied (Table 1). In the experimental model using BALB/c mice, it was observed that IL-17 contributes to the inflammation. On the other hand, an increase in IL-4 receptor activation causes the reduction of IL-17 and enhances the expression of insulin-like growth factor-1 (IGF-1) and IL-10, with consequent AAM activation and tissue repair. These data highlight the essential role of Th2 cytokines and AAMs in limiting lung damage [48].

Another study using FIZZ/Retnla gene KO mice, a Th2-inducible gene, showed that there was greater lung and liver damage in $Retnla^{-/-}$ mice infected with *N. brasiliensis* or *S. mansoni*, concomitant with exacerbation of fibrogenesis and increased IL-4 and IL-13 production, which in turn reduced parasite burden. These data suggest that the Retnla gene downregulates the Th2 immune response and suppresses resistance to nematode infection, granulomatous inflammation, and fibrosis [19].

The role of YM-1 during *N. brasiliensis* infection was also investigated. Sutherland et al., using neutralizing antibodies against YM-1 in an *in vivo* model, observed that YM-1 neutralization caused a decrease in neutrophils from bronchoalveolar lavage and lung tissue at 2 and 4 days after infection, followed by less inflammation but an increase in macrophages that was associated with lung healing [50]. Thus, even in the absence of YM-1, AAMs can still fulfill their repair function, and YM-1 appears to be implicated in neutrophilia and acute lung damage. However, other studies have confirmed that neutrophils are key elements in parasite neutralization and mediators of repair through AAM activation [51].

Other cells associated with tissue repair during *N. brasiliensis* infection include ILC2s, which are mainly induced by IL-25 and IL-33 [19] and to a lesser extent by IL-9, and appear to play an autocrine role amplifying ILC2s. The study was carried out on a model of $IL\text{-}9R^{-/-}$ mice, in which $IL\text{-}9R^{-/-}$ mice showed a significant decrease in ILC2s, IL-5, IL-13, and amphiregulin (a member protein of the epidermal growth factor family that promotes bronchoalveolar epithelium repair). Such a decrease was correlated with deficient tissue repair in the absence of IL-9. The tissue repair deficiency observed in this experimental setting was associated with a decrease in AAM markers, particularly Arg-1, Retnla, and YM-1, suggesting that ILC2s may induce AAMs

Table 1: Alternatively activated macrophages (AAMs) in the repair process during tissue migration and resident helminthes.

Helminth	Experimental model and mice strain	AAMs role	Ref.
Nippostrongylus brasiliensis	BALB/c	IGF-1 production is increased by IL-4, inducing AAM activation followed by tissue repair	[48]
	FIZZ1/Retnla$^{-/-}$	The Retnla gene downregulates the Th2 immune response and suppresses resistance to nematode infection, granulomatous inflammation, and fibrosis	[49]
	BALB/c	In the absence of YM-1, AAMs can still fulfill their repair function	[50]
	BALB/c	Neutrophils are key elements in parasite neutralization and are mediators of repair through AAM activation	[51]
	IL-9$^{-/-}$	ILC2s may induce AAMs, which mediate adequate tissue repair	[52]
	STAT6$^{-/-}$ and Arg-1$^{fl/fl}$	ILC2s constitutively express Arg-1, and they can repair lung tissue during acute inflammation in the absence of AAMs	[20]
Toxocara canis	STAT6$^{-/-}$	STAT6 absence may cause delayed wound healing by the reduction of the AAM population	[54]
Schistosoma mansoni	LysM^{cre}IL-4$^{-/lox}$ and IL-4$^{-/-}$	Polarization to the Th1 immune response, associated with CAM activation and NOS production, is related to hepatic damage and death	[57]
	IL-4Ra$^{lox/\Delta}$LysM^{Cre}	AAMs are necessary for pathogenic Th1/CAM suppression	[58]
	FLH$^{-/-}$	The absence of FIH1 induces Th1 polarization, which is associated with an increase in granuloma hepatic formation	[62]
	Arg-1$^{-/-}$	Arg-1 production by AAMs may play an important role in *S. mansoni* infection control and diminish intestinal damage	[63]
Heligmosomoides polygyrus bakeri	Jɪ$^{-/-}$ and FcRγ$^{-/-}$	Antibodies cause AAM activation, enhancing the expression of genes associated with the repair mechanisms	[43]
	C57BL/6 and Cx3Cr1$^{GFP/+}$	Infection increases the expression of Ym-1, RELM-α, and CD206, enhancing collagen deposition and fibrosis in heart tissue	[68]
Trichinella spiralis	Ob/ob and C57BL/6	AAMs mediate inflammation in adipose tissue, insulin resistance, and glucose control	[69]
	BALB/c	The rTsP53 protein appears to be beneficial during colitis, with an increase in AAMs	[70]
Taenia crassiceps	BALB/c	AAMs have immunomodulatory effects in experimental colitis and colon cancer models	[71, 72]
	C57BL/6	A decrease in inflammation and EAE symptoms is associated with AAM activation	[73]
Trichuris muris	Arg-1$^{fl/fl}$, Tie2-cre, and C57BL/6	Arg-1 is dispensable for tissue repair, but its absence is not related to damage	[74]

to mediate the tissue repair process [52]. Nevertheless, there is recent evidence indicating that ILC2s constitutively express Arg-1 [20]. Moreover, Monticelli et al. demonstrated that ILC2s are the major source of Arg-1 in the lungs even more than alveolar macrophages in basal conditions; however, their role in lung inflammation is controversial [15].

7.2. Toxocara canis. Toxocariasis is a worldwide zoonotic parasitic disease caused by the nematode *T. canis*. In humans, the infection is caused by accidental ingestion of embryonated eggs from contaminated soil: the eggs hatch, and the liberated larvae migrate to different organs, producing various disorders. Murine models have shown the presence of transitory hemorrhagic pulmonary lesions associated with strong Th2 responses and heavy parasite burdens. During *T. canis* infection, there is predominance of a Th2 immune response, characterized by the production of IL-4, IL-5, IL-13, and immunoglobulin subclasses IgG1 and IgE, as well as an increase in peripheral blood eosinophils and eosinophilic granuloma in the lung and liver [53].

Although *T. canis* can migrate through diverse organs and the immune response described during this infection is prone to induce AAM, there are very few studies assessing their role in tissue repair. Only one study has evaluated the possible role of AAM markers during this infection. In this study, STAT6 $^{-/-}$ and WT mice were challenged orally with *T. canis* larvae, where longer persistence of hemorrhagic pulmonary lesions and inflammation in STAT6$^{-/-}$ mice was observed to be associated with a weak Th2 immune response as a consequence of the inhibition of the STAT6 signaling pathway. By contrast, WT mice displayed strong Th2 immune responses, associated with high levels of IgG1, IgE, and IL-4 and the presence of AAM markers in lung tissue. Additionally, these WT mice resolved the lung lesions faster than STAT6 $^{-/-}$ mice. Interestingly, STAT6 $^{-/-}$ mice displayed significantly lower parasite loads. These data suggest that the severity in lung damage and persistence of lesions are associated with the absence of AAMs, as suggested for other helminth infections (Table 1) [54]. Strong inflammatory lung reactions in human toxocariasis have been well described, which may trigger chronic hypersensitivity mediated by an eosinophilic environment and granulomatous inflammation. Eosinophilic granuloma enables pathogen neutralization; however, it may have deleterious effects on the host, and AAM may play an important role in decreasing inflammation and favoring tissue repair, although its role in toxocariasis is yet to be determined [33, 55, 56]. Similarly, studies on the role of ILC2s in tissue repair during toxocariasis are lacking.

7.3. Schistosoma mansoni. AAMs are an essential cell type during schistosomiasis (Table 1), which are involved in the reduction of tissue inflammation and associated injury triggered by *S. mansoni* eggs deposited in liver tissue. To prove the role of this cell type, Herbert et al. used an experimental model of LysM^{cre}IL-4 ^{flox} and IL-4 deficient mice, both with impaired activation of AAMs and with the enhanced ability to induce CAM expressing iNOS2. The authors also observed that WT mice had smaller liver granulomas and higher expression of Arg-1 than LysM^{cre}IL-4$^{-/flox}$ and IL-4 deficient mice [57]. Similar results were published by Vannella et al. using IL-4Rα$^{flox/\Delta}$LysM^{Cre} mice, concluding that AAMs are necessary to suppress pathogenic Th1/CAM responses without a significant impact on fibrosis, although fibrosis was slightly higher in IL-4Rα$^{flox/\Delta}$LysM^{Cre} mice [58].

By contrast, previous studies have shown that IL-4 and IL-13 (Th2 type response) may play dual roles in lung granuloma formation, which is necessary for *S. mansoni* egg containment, suggesting that Th2 immune responses may produce tissue damage rather than repair. For example, IL-13 induces tissue eosinophilia and high levels of IgE enhancing lung granuloma formation, whereas IL-13 blockage in mice was accompanied by changes in eosinophil accumulation and reduced granuloma size [59]. This is in line with another study that has established the fact that IL-13 exhibits chemotactic activity for human eosinophils; therefore, schistosome granulomas are rich in eosinophils [60]. Thus, damaged tissue is associated with eosinophil recruitment without participation of AAM, although both cell types are part of the Th2 immune response [59]. However, it has been recently observed that AAMs are also important in maintaining local Th2 responses in general and IL-13 production in particular during *S. mansoni*-induced granuloma formation, as demonstrated by partial AMM depletion that overall reduces lung fibrosis and pulmonary inflammation, as described by Borthwick et al. [61].

In an *in vitro* study, using bone marrow macrophages differentiated with IL-10 and IL-4 to AAMs, upregulation of FHL2 (a protein structural domain, also called LIM) was observed. However, when the bone marrow macrophages from FHL2$^{-/-}$ mice were similarly stimulated, the AAM genes were downregulated, and CAM markers seemed to be upregulated, proving the expression of FHL2 induced in mouse marrow-derived macrophages following stimulation with AAM-inducer cytokines. To prove the importance of FHL2 in AAM activation, FHL2$^{-/-}$ mice were challenged with *S. mansoni* showing higher numbers of granulomas and reduced expression of AAM markers, which correlate with an enhanced Th1 immune response. These data suggest a role for FHL2 in the pathogenesis of pulmonary granulomatous inflammation through AAM polarization and Th1/Th2 balance [62].

During intestinal schistosomiasis by *S. mansoni*, the use of *S*-(2-boronoethyl)-L-cysteine (BEC), an Arg-1, and Arg-2 antagonist, was related to impaired elimination of *S. mansoni* eggs that correlated with an increase in disease severity and mortality compared with that in nontreated mice. In the same study, now using Arg-1$^{-/-}$ mice, the authors observed hemorrhagic lesions in the intestinal mucosa that were not observed in WT mice. These data confirmed that Arg-1 production by AAMs is important for both *S. mansoni* infection control and reducing intestinal damage, and the absence of Arg-1 causes Th1 polarization associated with a proinflammatory cytokine profile [63].

Evidence that a decreased Th1 immune response and a reduced number of CAMs and therefore indirect stimulation

of AAM contribute to repair mechanisms has been shown in CD14 (a TLR-4 coreceptor) deficient mice. These mice have fewer and smaller hepatic granulomas and an increase in $CD4^+IL\text{-}4$, IL-5, IL-13$^+$, and $CD4^+Foxp3^+IL\text{-}10^+$ cells that correlate with collagen deposition and wound healing. This effect was associated with STAT6 signaling, suggesting that the absence of CD14 has an impact on the IL4α/STAT6 pathway and macrophage polarization during infection [64, 65]. Moreover, the inhibition of cytokines or factors related to AAM polarization causes a decrease in the protective role of AMM. In conclusion, most data point out these cell types as vital for the successful repair of lesions during schistosomiasis [66, 67].

7.4. Heligmosomoides polygyrus bakeri. As mentioned above, antibodies also mediate AAM activation. This fact has been observed during *H. polygyrus bakeri* infection (Table 1). Esser-von Bieren et al. described that complement-dependent antibodies that bind to FcRγ cause macrophage adherence to *H. polygyrus* larvae *in vitro*, immobilizing the parasite, triggering macrophage reprogramming, and enhancing the expression of genes associated with repairing mechanisms. Such mechanisms were independent of the IL-4Rα signaling pathway, suggesting a different AAM activation mechanism [43].

Other studies have proven that cardiac resident macrophages, in the absence of infection, expressed classical (IL-1β, TNF-α, and CCR2) and alternative (Ym-1, Arg-1, RELM-α, and IL-10) markers. Nevertheless, during *H. polygyrus* infection, there is an increase in the expression of Ym-1, RELM-α, and CD206 and enhanced collagen deposition, causing fibrosis in heart tissue. Although *H. polygyrus* is a local migratory tissue parasite (which penetrates submucosal layer of the small intestine to the muscularis externa and later towards the lumen), polarization of AAM is induced by the immunologic activation of infection [68]. However, there is a lack of information regarding the role of AAMs in cardiac tissue repair. Although it has been suggested that fibrosis is a mechanism of tissue repair, it is still necessary to determine the positive or negative fibrotic effect on the heart during *H. polygyrus* infection. However, the idea that an intestinal helminth infection could have an effect on an organ as the heart is very interesting to explore.

7.5. Trichinella spiralis. During trichinellosis by *T. spiralis*, it has been reported that macrophages are important mediators of inflammation in adipose tissue, insulin resistance, and glucose control (Table 1). Therefore, the role of AAMs in obesity has been studied in the context of helminth infection. Using an experimental model of obese mice infected with *T. spiralis*, the induction of AAMs triggered by helminth infection led to decreased glucose intolerance and consequent lowering of the blood glucose levels which was associated with AAM markers such as Arg-1, CD206, and IL-10, as well as adipocyte death [69]. These results suggest that AAMs, which are induced by *T. spiralis* infection, have a beneficial role during obesity through the regulation of the inflammatory process in adipose tissue.

In other inflammatory diseases, such as colitis (Table 1), it has been observed that excretory/secretory (ES) proteins produced by these parasites have immunomodulatory effects. Among those ES proteins produced by *T. spiralis*, the recombinant 53 kDa protein rTsP53 was found to polarize the immune response to the Th2 phenotype. Using this protein during experimental colitis caused a Th2 immune response that correlates to reduced inflammation and the enhanced expression of the AAM markers Arg-1, FIZZ-1, TGF-β, and IL-10 [70]. These anti-inflammatory effects triggered by rTsP53 appear to be helpful in repairing tissue during colitis.

7.6. Taenia crassiceps. *T. crassiceps* is a cestode that has been extensively studied. These parasites induce a population of AAMs with suppressive activity and have been shown to have immunomodulatory effects on experimental colitis and colon cancer models (Table 1) [71, 72]. In this regard, AAMs play a central role in modulating both colonic inflammation and colitis-associated tumorigenesis. During experimental colitis, it has been shown that *T. crassiceps* infection induces the expression of Arg-1, YM-1, and FIZZ-1, which is related to increased collagen deposition in the intestine that does not cause fibrosis but diminishes intestinal inflammation and hemorrhage. Moreover, when AAMs isolated from *T. crassiceps*-infected mice were adoptively transferred to colitic mice, these cells could ameliorate ongoing colitis [71, 72].

A similar effect was observed in experimental autoimmune encephalomyelitis (EAE), in which the presence of *T. crassiceps* causes a decrease in inflammation and symptoms of encephalomyelitis with repair in the spinal cord. Such effects were associated with anti-inflammatory cytokine production and expression of AAM markers [73]; together, these data indicate that sometimes helminth infections generate improved side effects.

7.7. Trichuris muris. There is limited information regarding the role of AAMs during infection of the intestinal nematode *Trichuris muris* (Table 1), but it has been established that it induces a Th2 immune response and therefore induction of AAMs. The only study that has investigated the role of AAM during *T. muris* infection showed that Arg-1 is dispensable for tissue repair, and its absence was not related to more damage [74]. However, it was not determined whether other mechanisms associated with AAM or not were responsible for tissue healing.

8. Concluding Remarks

The type 2 immune response has evolved to direct the wound-healing machinery not only to repair and remodel tissue but also to mediate the containment, destruction, or expulsion of helminths. Both effects have been associated with the presence of AAMs; particularly, the issue related to tissue repair has also been related to other mechanisms independent of AAMs, such as ILC2s and epithelial cells from lung tissue that constitutively express Arg-1 for collagen production. Consequently, those cells could also be involved in tissue repair. Given such information, another way to

prove the importance of AAMs and other sources of collagen production in the repair process during helminth tissue migration could be depleting macrophages from lung tissue and measuring Arg-1 and collagen deposition associated with ILC2s or pulmonary epithelial cells. Even though there has been great progress in the understanding of AAMs functions and their role in tissue repair, a full depletion of AAMs from lung tissue has not been achieved, and there are still points of uncertainty and controversies that must be resolved in the future.

Competing Interests

The authors have no competing financial or commercial interests.

Acknowledgments

This work was supported by Grant 167799 from CONACYT. Berenice Faz-López is a recipient of a Ph.D. fellowship from CONACYT, and this work was performed in partial fulfillment of the requirements for the Ph.D. Programa de Doctorado en Ciencias Biomédicas, FES Iztacala, Universidad Nacional Autónoma de México.

References

[1] C. Romero Núñez, G. D. Mendoza Martínez, S. Yañez Arteaga, M. Ponce Macotela, P. Bustamante Montes, and N. Ramírez Durán, "Prevalence and risk factors associated with *Toxocara canis* infection in children," *The Scientific World Journal*, vol. 2013, Article ID 572089, 4 pages, 2013.

[2] J. M. Craig and A. L. Scott, "Helminths in the lungs," *Parasite Immunology*, vol. 36, no. 9, pp. 463–474, 2014.

[3] R. Shah and S. Chakrabarti, "Neuropsychiatric manifestations and treatment of disseminated neurocysticercosis: a compilation of three cases," *Asian Journal of Psychiatry*, vol. 6, no. 4, pp. 344–346, 2013.

[4] J. H. Rockey, T. John, J. J. Donnelly, D. F. McKenzie, B. K. Stromberg, and E. J. Soulsby, "In vitro interaction of eosinophils from ascarid-infected eyes with Ascaris suum and Toxocara canis larvae," *Investigative Ophthalmology and Visual Science*, vol. 24, no. 10, pp. 1346–1357, 1983.

[5] H. Bächli, J. C. Minet, and O. Gratzl, "Cerebral toxocariasis: a possible cause of epileptic seizure in children," *Child's Nervous System*, vol. 20, no. 7, pp. 468–472, 2004.

[6] D. H. Esposito, A. Stich, L. Epelboin et al., "Acute muscular sarcocystosis: an international investigation among ill travelers returning from Tioman Island, Malaysia, 2011-2012," *Clinical Infectious Diseases*, vol. 59, no. 10, pp. 1401–1410, 2014.

[7] J. R. Lambertucci, A. Rayes, J. C. Serufo et al., "Visceral larva migrans and tropical pyomyositis: a case report," *Revista do Instituto de Medicina Tropical de Sao Paulo*, vol. 40, no. 6, pp. 383–385, 1998.

[8] B. D. Robertson, A. T. Bianco, J. H. Mckerrow, and R. M. Maizels, "*Toxocara canis*: proteolytic enzymes secreted by the infective larvae *in vitro*," *Experimental Parasitology*, vol. 69, no. 1, pp. 30–36, 1989.

[9] J. H. McKerrow, P. Brindley, M. Brown, A. A. Gam, C. Staunton, and F. A. Neva, "Strongyloides stercoralis: identification of a protease that facilitates penetration of skin by the infective larvae," *Experimental Parasitology*, vol. 70, no. 2, pp. 134–143, 1990.

[10] C. Trap and P. Boireau, "Les protéases chez les helminthes," *Veterinary Research*, vol. 31, no. 5, pp. 461–471, 2000.

[11] P. A. Jimenez and S. E. Jimenez, "Tissue and cellular approaches to wound repair," *American Journal of Surgery*, vol. 187, no. 5, 2004.

[12] A. J. Singer and R. A. F. Clark, "Cutaneous wound healing," *The New England Journal of Medicine*, vol. 341, no. 10, pp. 738–746, 1999.

[13] R. J. McAnulty, "Fibroblasts and myofibroblasts: their source, function and role in disease," *International Journal of Biochemistry and Cell Biology*, vol. 39, no. 4, pp. 666–671, 2007.

[14] S. J. Forbes and N. Rosenthal, "Preparing the ground for tissue regeneration: from mechanism to therapy," *Nature Medicine*, vol. 20, no. 8, pp. 857–869, 2014.

[15] L. A. Monticelli, M. D. Buck, A. Flamar et al., "Arginase 1 is an innate lymphoid cell intrinsic metabolic checkpoint controlling type 2 inflammation," *Nature Immunology*, vol. 17, no. 6, pp. 656–665, 2016.

[16] G. Gabbiani, "The myofibroblast in wound healing and fibrocontractive diseases," *The Journal of Pathology*, vol. 200, no. 4, pp. 500–503, 2003.

[17] S. Gordon and F. O. Martinez, "Alternative activation of macrophages: mechanism and functions," *Immunity*, vol. 32, no. 5, pp. 593–604, 2010.

[18] M. D. Taylor, N. van der Werf, and R. M. Maizels, "T cells in helminth infection: the regulators and the regulated," *Trends in Immunology*, vol. 33, no. 4, pp. 181–189, 2012.

[19] Y. Huang and W. E. Paul, "Inflammatory group 2 innate lymphoid cells," *International Immunology*, vol. 28, no. 1, pp. 23–28, 2016.

[20] J. K. Bando, J. C. Nussbaum, H.-E. Liang, and R. M. Locksley, "Type 2 innate lymphoid cells constitutively express arginase-I in the naïve and inflamed lung," *Journal of Leukocyte Biology*, vol. 94, no. 5, pp. 877–884, 2013.

[21] K. A. Ravin and M. Loy, "The eosinophil in infection," *Clinical Reviews in Allergy & Immunology*, vol. 50, no. 2, pp. 214–227, 2016.

[22] C. A. Behm and K. S. Ovington, "The role of eosinophils in parasitic helminth infections: insights from genetically modified mice," *Parasitology Today*, vol. 16, no. 5, pp. 202–209, 2000.

[23] D. Masure, J. Vlaminck, T. Wang et al., "A role for eosinophils in the intestinal immunity against infective *Ascaris suum* larvae," *PLoS Neglected Tropical Diseases*, vol. 7, no. 3, Article ID e2138, 2013.

[24] A. M. Galioto, J. A. Hess, T. J. Nolan, G. A. Schad, J. J. Lee, and D. Abraham, "Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval Strongyloides stercoralis in mice," *Infection and Immunity*, vol. 74, no. 10, pp. 5730–5738, 2006.

[25] D. A. Holmes, J.-H. Yeh, D. Yan, M. Xu, and A. C. Chan, "Dusp5 negatively regulates IL-33 mediated eosinophil survival and function," *The EMBO Journal*, vol. 34, no. 2, pp. 218–235, 2015.

[26] J. P. Hewitson, K. J. Filbey, J. Esser von Bieren et al., "Concerted activity of IgG1 antibodies and IL-4/IL-25-dependent effector cells trap helminth larvae in the tissues following vaccination with defined secreted antigens, providing sterile immunity to challenge infection," *PLoS Pathogens*, vol. 11, no. 3, Article ID e1004676, pp. 1–22, 2015.

[27] M. Takamoto, K. S. Ovington, C. A. Behm, K. Sugane, I. G. Young, and K. I. Matthaei, "Eosinophilia, parasite burden and lung damage in *Toxocara canis* infection in C57Bl/6 mice genetically deficient in IL-5," *Immunology*, vol. 90, no. 4, pp. 511–517, 1997.

[28] S. Hokibara, M. Takamoto, A. Tominaga, K. Takatsu, and K. Sugane, "Marked eosinophilia in interleukin 5 transgenic mice fails to prevent *Trichinella spiralis* infection," *The Journal of Parasitology*, vol. 83, no. 6, pp. 1186–1189, 1997.

[29] A. D. Klion and T. B. Nutman, "The role of eosinophils in host defense against helminth parasites," *Journal of Allergy and Clinical Immunology*, vol. 113, no. 1, pp. 30–37, 2004.

[30] H. Kubo, D. A. Loegering, C. R. Adolphson, and G. J. Gleich, "Cytotoxic properties of eosinophil granule major basic protein for tumor cells," *International Archives of Allergy and Immunology*, vol. 118, no. 2–4, pp. 426–428, 1999.

[31] M. K. Samoszuk, A. Petersen, F. Gidanian, and C. Rietveld, "Cytophilic and cytotoxic properties of human eosinophil peroxidase plus major basic protein," *American Journal of Pathology*, vol. 132, no. 3, pp. 455–460, 1988.

[32] G. J. Gleich, E. Frigas, D. A. Loegering, D. L. Wassom, and D. Steinmuller, "Cytotoxic properties of the eosinophil major basic protein," *Journal of Immunology*, vol. 123, no. 6, pp. 2925–2927, 1979.

[33] A. Malheiro, P. F. Anibal, O. A. Martins Filho et al., "pcDNA IL-12 vaccination blocks eosinophilic inflammation but not airway hyperresponsiveness following murine *Toxocara canis* infection," *Vaccine*, vol. 26, no. 3, pp. 305–315, 2008.

[34] P. K. Mishra, Q. Li, L. E. Munoz et al., "Reduced leukocyte infiltration in absence of eosinophils correlates with decreased tissue damage and disease susceptibility in AdIL6ATA mice during murine neurocysticercosis," *PLoS Neglected Tropical Diseases*, vol. 10, no. 6, Article ID e0004787, 2016.

[35] W. C. Gause, T. A. Wynn, and J. E. Allen, "Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths," *Nature Reviews Immunology*, vol. 13, no. 8, pp. 607–614, 2013.

[36] D. Date, R. Das, G. Narla, D. I. Simon, M. K. Jain, and G. H. Mahabeleshwar, "Kruppel-like transcription factor 6 regulates inflammatory macrophage polarization," *The Journal of Biological Chemistry*, vol. 289, no. 15, pp. 10318–10329, 2014.

[37] M. G. Nair, I. J. Gallagher, M. D. Taylor et al., "Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells," *Infection and Immunity*, vol. 73, no. 1, pp. 385–394, 2005.

[38] P. Bhattacharya, R. Dey, P. K. Dagur et al., "Genetically modified live attenuated Leishmania donovani parasites induce innate immunity through classical activation of macrophages that direct the Th1 response in mice," *Infection and Immunity*, vol. 83, no. 10, pp. 3800–3815, 2015.

[39] M. Munder, "Arginase: an emerging key player in the mammalian immune system," *British Journal of Pharmacology*, vol. 158, no. 3, pp. 638–651, 2009.

[40] E. Song, N. Ouyang, M. Hörbelt, B. Antus, M. Wang, and M. S. Exton, "Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts," *Cellular Immunology*, vol. 204, no. 1, pp. 19–28, 2000.

[41] T. N. Holcomb, R. C. Kabakoff, B. Chan et al., "FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family," *The EMBO Journal*, vol. 19, no. 15, pp. 4046–4055, 2000.

[42] T. Liu, S. M. Dhanasekaran, H. Jin et al., "FIZZ1 stimulation of myofibroblast differentiation," *American Journal of Pathology*, vol. 164, no. 4, pp. 1315–1326, 2004.

[43] J. Esser-von Bieren, I. Mosconi, R. Guiet et al., "Antibodies trap tissue migrating helminth larvae and prevent tissue damage by driving IL-4Rα-independent alternative differentiation of macrophages," *PLoS Pathogens*, vol. 9, no. 11, Article ID e1003771, 2013.

[44] H. Min, Y. H. Jang, I. Cho, S. Yu, and S. J. Lee, "Alternatively activated brain infiltrating macrophages facilitate recovery from collagenase-induced intracerebral hemorrhage," *Molecular Brain*, vol. 9, no. 1, article 42, 2016.

[45] F. Qian, J. Deng, Y. G. Lee et al., "The transcription factor PU.1 promotes alternative macrophage polarization and asthmatic airway inflammation," *Journal of Molecular Cell Biology*, vol. 7, no. 6, pp. 557–567, 2015.

[46] B. Csóka, B. Koscsó, G. Töro et al., "A2B Adenosine receptors prevent insulin resistance by inhibiting adipose tissue inflammation via maintaining alternative macrophage activation," *Diabetes*, vol. 63, no. 3, pp. 850–866, 2014.

[47] M. Shiraishi, Y. Shintani, H. Ishida et al., "Alternatively activated macrophages determine repair of the infarcted adult murine heart," *The Journal of Clinical Investigation*, vol. 126, no. 6, pp. 2151–2166, 2016.

[48] F. Chen, Z. Liu, W. Wu et al., "An essential role for TH2 type responses in limiting acute tissue damage during experimental helminth infection," *Nature Medicine*, vol. 18, no. 2, pp. 260–266, 2012.

[49] J. T. Pesce, T. R. Ramalingam, M. S. Wilson et al., "Retnla (Relmα/Fizz1) suppresses helminth induced Th2 type immunity," *PLoS Pathogens*, vol. 5, no. 4, article e1000393, 2009.

[50] T. E. Sutherland, N. Logan, D. Rückerl et al., "Chitinase-like proteins promote IL-17 mediated neutrophilia in a tradeoff between nematode killing and host damage," *Nature Immunology*, vol. 15, no. 12, pp. 1116–1125, 2014.

[51] F. Chen, W. Wu, A. Millman et al., "Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion," *Nature Immunology*, vol. 15, no. 10, pp. 938–946, 2014.

[52] J.-E. Turner, P. J. Morrison, C. Wilhelm et al., "IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation," *The Journal of Experimental Medicine*, vol. 210, no. 13, pp. 2951–2965, 2013.

[53] R. Pinelli, S. Brandes, J. Dormans, M. Romille, C. M. Hamilton, and J. V. der Giessen, "Toxocara canis: effect of inoculum size on pulmonary pathology and cytokine expression in BALB/c mice," *Experimental Parasitology*, vol. 115, no. 1, pp. 76–82, 2007.

[54] B. Paz-López, Y. Ledesma-Soto, Y. Romero-Sánchez, E. Calleja, P. Martínez-Lahat, and L. I. Terrazas, "Signal transducer and activator of transcription factor 6 signaling contributes to control host lung pathology but favors susceptibility against toxocara canis infection," *BioMed Research International*, vol. 2013, Article ID 696343, 11 pages, 2013.

[55] D. Nagy, O. Bede, J. Danka, Z. Szénási, and S. Sipka, "Analysis of serum cytokine levels in children with chronic cough associated with *Toxocara canis* infection," *Parasite Immunology*, vol. 34, no. 12, pp. 581–588, 2012.

[56] R. G. Bahnea, I. Cojocaru, C. Ripă, M. C. Luca, A. Ivans, and M. Luca, "Toxocariasis respiratory manifestations in cases hospitalized in the Paediatric Diseases Clinic of Iași, between 2005–2008," *Revista Medico Chirurgicala a Societatii de Medici si Naturalisti din Iasi*, vol. 113, no. 4, pp. 1099–1101, 2009.

[57] D. R. Herbert, C. Hölscher, M. Mohrs et al., "Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology," *Immunity*, vol. 20, no. 5, pp. 623–635, 2004.

[58] K. M. Vannella, L. Barron, L. A. Borthwick et al., "Incomplete deletion of IL-4Rα by LysM(Cre) reveals distinct subsets of M2 macrophages controlling inflammation and fibrosis in chronic schistosomiasis," *PLoS pathogens*, vol. 10, no. 9, Article ID e1004372, 2014.

[59] M. G. Chiaramonte, L. R. Schopf, T. Y. Neben, A. W. Cheever, D. D. Donaldson, and T. A. Wynn, "IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by Schistosoma mansoni eggs," *Journal of Immunology*, vol. 162, no. 2, pp. 920–930, 1999.

[60] S. Horie, Y. Okubo, M. Hossain et al., "Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis," *Internal Medicine*, vol. 36, no. 3, pp. 179–185, 1997.

[61] L. A. Borthwick, L. Barron, K. M. Hart et al., "Macrophages are critical to the maintenance of IL-13-dependent lung inflammation and fibrosis," *Mucosal Immunology*, vol. 9, no. 1, pp. 38–56, 2016.

[62] K. Kurakula, M. Vos, M. van Eijk, H. H. Smits, and C. J. M. de Vries, "LIM-only protein FHL2 regulates experimental pulmonary *Schistosoma mansoni* egg granuloma formation," *European Journal of Immunology*, vol. 45, no. 11, pp. 3098–3106, 2015.

[63] D. R. Herbert, T. Orekov, A. Roloson et al., "Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis," *Journal of Immunology*, vol. 184, no. 11, pp. 6438–6446, 2010.

[64] S. Tundup, L. Srivastava, T. Nagy, and D. Harn, "CD14 influences host immune responses and alternative activation of macrophages during Schistosoma mansoni infection," *Infection and Immunity*, vol. 82, no. 8, pp. 3240–3251, 2014.

[65] R. Edukulla, B. Singh, A. G. Jegga, V. Sonlake, S. R. Dillon, and S. K. Madala, "Th2 cytokines augment IL 31/IL 31RA interactions via STAT6-dependent IL-31RA expression," *The Journal of Biological Chemistry*, vol. 290, no. 21, pp. 13510–13520, 2015.

[66] R. Rani, M. B. Jordan, S. Divanovic, and D. R. Herbert, "IFN-γ driven IDO production from macrophages protects IL-4Rα deficient mice against lethality during schistosoma mansoni infection," *American Journal of Pathology*, vol. 180, no. 3, pp. 2001–2008, 2012.

[67] M. Nascimento, S. C. Huang, A. Smith et al., "Ly6Chi monocyte recruitment is responsible for Th2 associated host-protective macrophage accumulation in liver inflammation due to schistosomiasis," *PLoS Pathogens*, vol. 10, no. 8, article e1004282, 2014.

[68] K. J. Mylonas, S. J. Jenkins, R. B. P. Castellan et al., "The adult murine heart has a sparse, phagocytically active macrophage population that expands through monocyte recruitment and adopts an 'M2' phenotype in response to Th2 immunologic challenge," *Immunobiology*, vol. 220, no. 7, pp. 924–933, 2015.

[69] H. Okada, T. Ikeda, K. Kajita et al., "Effect of nematode *Trichinella* infection on glucose tolerance and status of macrophage in obese mice," *Endocrine Journal*, vol. 60, no. 11, pp. 1241–1249, 2013.

[70] L. Du, H. Tang, Y. Ma et al., "The protective effect of the recombinant 53 kDa protein of *Trichinella spiralis* on experimental colitis in mice," *Digestive Diseases and Sciences*, vol. 56, no. 10, pp. 2810–2817, 2011.

[71] S. León-Cabrera, B. E. Callejas, Y. Ledesma-Soto et al., "Extraintestinal helminth infection reduces the development of colitis-associated tumorigenesis," *International Journal of Biological Sciences*, vol. 10, no. 9, pp. 948–956, 2014.

[72] Y. Ledesma-Soto, B. E. Callejas, C. A. Terrazas et al., "Extraintestinal helminth infection limits pathology and proinflammatory cytokine expression during DSS induced ulcerative colitis: a role for alternatively activated macrophages and prostaglandins," *BioMed Research International*, vol. 2015, Article ID 563425, 17 pages, 2015.

[73] J. L. Reyes, A. F. Espinoza-Jiménez, M. I. González, L. Verdin, and L. I. Terrazas, "Taenia crassiceps infection abrogates experimental autoimmune encephalomyelitis," *Cellular Immunology*, vol. 267, no. 2, pp. 77–87, 2011.

[74] R. Bowcutt, L. V. Bell, M. Little et al., "Arginase 1 expressing macrophages are dispensable for resistance to infection with the gastrointestinal helminth Trichuris muris," *Parasite Immunology*, vol. 33, no. 7, pp. 411–420, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 695343, 11 pages
http://dx.doi.org/10.1155/2013/695343

Hindawi

Research Article

Signal Transducer and Activator of Transcription Factor 6 Signaling Contributes to Control Host Lung Pathology but Favors Susceptibility against *Toxocara canis* Infection

Berenice Faz-López,[1,2] Yadira Ledesma-Soto,[1] Yolanda Romero-Sánchez,[3] Elsa Calleja,[4] Pablo Martínez-Labat,[2] and Luis I. Terrazas[1]

[1] *Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México (UNAM), Avenue de los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, MEX, Mexico City, DE, Mexico*
[2] *Laboratorio de Parasitología, Facultad de Estudios Superiores Cuautitlán, UNAM, 54714 Mexico City, DE, Mexico*
[3] *Laboratorio de Histopatología, Facultad de Estudios Superiores Cuautitlán, UNAM, 54714 Mexico City, DE, Mexico*
[4] *Carrera de Medicina, Facultad de Estudios Superiores Iztacala, UNAM, 54090 Mexico City, DE, Mexico*

Correspondence should be addressed to Luis I. Terrazas; literrazas@campus.iztacala.unam.mx

Received 10 August 2012; Accepted 29 October 2012

Academic Editor: Miriam Rodríguez Sosa

Using STAT6$^{-/-}$ BALB/c mice, we have analyzed the role of STAT6-induced Th2 response in determining the outcome of experimental toxocariasis caused by embryonated eggs of the helminth parasite *Toxocara canis*. Following *T. canis* infection wild-type BALE/c mice developed a strong Th2-like response, produced high levels of IgG1, IgE, and IL-4, recruited alternatively activated macrophages, and displayed a moderate pathology in the lungs; however, they harbored heavy parasite loads in different tissues. In contrast, similarly infected STAT6$^{-/-}$ BALB/c mice mounted a weak Th2-like response, did not recruit alternatively activated macrophages, displayed a severe pathology in the lungs, but efficiently controlled *T. canis* infection. These findings demonstrate that Th2-like response induced via STAT6-mediated signaling pathway mediates susceptibility to larval stage of *T. canis*. Furthermore, they also indicate that unlike most gastrointestinal helminths, immunity against larvae of *T. canis* is not mediated by a Th2-dominant response.

1. Introduction

Toxocariasis is a helminth infection considered as a zoonosis and is caused by the larvae of *Toxocara canis*; this parasite affects many paratenic hosts including humans. This infection is world-wide distributed mainly because its transmission is associated with domestic dogs. This disease is starting to be considered as a public health problem in Latin America and Asia [1, 2] but has been extended in the last few years to developed countries as shown by increased numbers of cases-report published more frequently [3–5]. Toxocariasis in humans results from accidental ingestion of *Toxocara*-embryonated eggs from excreta in the environment or by direct dog-to-person contact [6, 7]. Although larvae in muscle may be relatively symptom less, those in brain may cause serious neurological disorders [8]; these symptoms are believed to result from inflammation following degeneration of the parasite [9].

In the experimental model of murine toxocariasis, infection of inbred mice with *T. canis* induces a strong Th2 like response similar to that observed following infection with other helminthes such as *Nippostrongylus brasiliensis* and *Trichuris muris* [10–12]. While widely accepted, Th2 type response in clearing helminth infections has limitations [11, 13–17], its role in mediating protection against toxocariasis in paratenic hosts is not clear, mainly because reinfection

in toxocariasis favors parasite survival [18]. Recent advances in the immunobiology of *T. canis* indicate that regulatory mechanisms are raised after infection; mainly T regulatory cells have been involved in limiting pathologic damage by the inflammatory response [19]; however, there are few data regarding on mechanism of protection or susceptibility against such parasite.

Previous studies have found that some extraintestinal larvae from other helminthes such as *Taenia crassiceps and Trichinella spiralis* are apparently eliminated in infected mice by a Th1-mediated inflammatory response during early phase of infection [20–22]. Furthermore, both studies found that STAT6 was involved during early phase of infection in rendering them more susceptible to cysticercosis and trichinellosis, respectively. These findings suggest that while Th2 type response may be involved in mediating resistance on gastrointestinal helminth infections, this pathway may be involved in susceptibility to the larval stages of such parasites. Numerous studies using STAT6$^{-/-}$ mice have shown that STAT6-mediated IL-4/IL-13 signaling pathway is critical for Th2 differentiation [23]. For example, STAT6$^{-/-}$ mice fail to mount a significant Th2 response and cannot control worm burdens following infection with gastrointestinal helminth parasites [17, 24]. Conversely, STAT6$^{-/-}$ mice develop a Th1 like response and control infections caused by intracellular protozoan parasites such as *Leishmania mexicana* and *Trypanosoma cruzi* [25, 26] indicating that STAT6-mediated signaling pathway inhibits development of protective immunity by inhibiting a Th1 development.

The purpose of this study was to determine the role of a Th2 type response induced via STAT6 medicted signaling in the outcome of experimental murine toxocariasis caused by the L2 of the nematode *T. canis*. To approach this question, we compared the course of *T. canis* infection in STAT6$^{-/-}$ BALB/c mice (STAT6$^{-/-}$) with that in the wild-type BALB/c (STAT6$^{+/+}$) mice. In addition, we analyzed both the antibody and cytokine profiles in sera, as well as the phenotype of lung macrophages. Our data demonstrate that Th2-type response induced via STAT6-signaling pathway mediates susceptibility in toxocariasis.

2. Materials and Methods

2.1. Mice. Six-8-week-old male STAT6$^{-/-}$ and STAT6$^{+/+}$ mice in a genetic BALB/c background were originally purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, Maine, USA) and were maintained in a pathogen-free environment at the FES-Iztacala, U.N.A.M. animal facility in accordance with Institutional and National guidelines.

2.2. Isolation of Larvae and Eggs and Infection Protocol. Adult *T. canis* females' worms were isolated from the intestine of naturally infected puppies (<3 months). Isolation and embryonation of eggs were performed as follows: female worms were dissected and from the uterus eggs were isolated and putted into distilled water; then the mixture was centrifuged two times for 10 min at 2,000 xg in a solution of NaHCl at 1%. After removal of the supernatant, the sediment was two times washed in distilled water and placed into the solution of formalin at 1% in tissue flasks at 28°C for 1 month with gentle daily agitation until the end of embryonation which was controlled under the microscope.

2.3. Infection. Five hundred larvated eggs were intragastrically administered with a Foley tube to both STAT6$^{-/-}$ and STAT6$^{+/+}$ mice. Infected mice were sacrificed at days 5, 14, and 60 postinfection, and the parasites harvested from different tissues (lung, liver, brain, and muscle) were enumerated as described previously [27].

For histological evaluation of different tissues, animals were euthanized at indicated days. The liver, lung, brain, and muscle were removed and fixed in 4% formalin. Tissue samples were embedded in paraffin, and 5 μm sections were cut on a microtome and stained with hematoxylin and eosin for histological examination.

2.4. Cytokine Measurements. The IL-4, IL-12, and IFN-γ levels were quantified in mouse serum at the indicated point times. Antibody pairs were used according to the manufacturer's instruction (Peprotech México, México, DF).

2.5. Toxocara-Specific Antibody Level and Total IgE. Peripheral blood was collected at the indicated time points after Toxocara infection from tail snips. The blood was centrifuged at 2500 rpm for 10 min, and serum was collected and tested for Toxocara-specific IgG1 and IgG2a in antigen-coated plates (1 μg/mL). After an overnight incubation at 4°C, the plates were washed with PBS supplemented with 0.05% Tween 20 (Sigma, St. Louis, MO, USA) and blocked with PBS supplemented with 1% BSA (US Biological, Swampscott, MA, USA). Serial dilutions (starting from 1 : 100) of the serum samples were added to the plates. The bound antibodies were detected following incubation with HRP-conjugated rat anti-mouse IgG1 or IgG2a (Zymed, San Francisco, CA, USA). The reactions were developed with ABTS solution (Zymed) and read on a microplate reader at 405 nm (Multiskan Ascent, Thermo Labsystems). Results are expressed as the maximal sera dilution (endpoint titer) where OD was detected. Total IgE production was detected by Opt-ELISA from Biolegend.

2.6. Flow Cytometry. It has been previously shown that macrophages recruited by helminth parasites to the site of infection express alternatively activated and suppressive markers, such as mannose receptor (MR), PD1 ligand 1 (PD-L1), and PD-L2. To determine whether *T. canis*-infected mice recruit such population, flow cytometry was performed on lung exudates cells from *T. canis*-infected at different times after infection. Briefly, 5, 14, and 60 days after infection, lungs lavages were aseptically obtained, and 1 × 10^6 cells were incubated with anti-CD16 and anti-CD32 (Biolegend, San Diego, CA, USA) to block nonspecific antibody binding. The cells were then stained with APC-conjugated anti-F4/80,

FITC-conjugated anti-MR, PE-conjugated anti-PDL1, and PE-conjugated anti-Gr1 (all from Biolegend) and incubated for 30 min. at 4°C in FACS staining buffer (1% FBS, 0.5% sodium azide in PBS). The cells were analyzed using a FACSCalibur and Cell Quest software (Becton Dickinson).

2.7. RT-PCR. RNA was extracted from isolated spleen cells after different day's postinfection using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the isopropanol-chloroform technique. The RNA was quantified, and 5 μg of RNA was reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen). PCR reactions containing 5x PCR Buffer blue, 10 mM dNTP, 40 nM each forward and reverse primers, 1 U Taq DNA polymerase (Sacace Biotechnologies, Italy), and 2 μL of the cDNA were prepared in a 25 μL final volume. The PCR conditions were elsewhere described [28]. Briefly, consisted of an initial denaturation step at 95°C for 5 min; 35 cycles of 95°C for 10 s, the indicated melting temperature for 50 s and 72°C for 40 s; a final extension step at 72°C for 4 min in a thermal cycler (Corbett Research, Australia). The amplified products were mixed with loading buffer containing SYBR green and observed in a 1.5% agarose gel with the Fujifilm FLA 5000 scanner (Fuji, Japan) using the image reader V2.1 software to capture the images.

2.8. Statistical Analysis. Comparisons between wild type ($STAT6^{+/+}$) and $STAT6^{-/-}$ groups considered in this work were made using student's unpaired t test. $P < 0.05$ was considered significant. The statistical significance of the sera titers were determined by nonparametric tests using Mann-Whitney U-Wilcoxon Rank.

3. Results and Discussion

It is largely accepted that the Th2-like response induced via STAT6-mediated signaling pathway (through IL-4/IL-13 receptors) plays a critical role in mediating protective immunity against most helminthes [12, 17, 28]. For example, STAT6-mediated signaling has been shown to promote protective immunity against gastrointestinal helminthes such as *Trichinella spiralis*, *N. brasiliensis*, and *Hymenolepis diminuta* [12, 17, 29, 30]. However, the role for many molecules associated with the immune response, including STAT6, during infection with *Toxocara canis* is unknown. Here we analyzed the potential role of STAT6 in modulating immunity against this nematode parasite. One of the first organs that *T. canis* larvae reach early after infection is the lungs, in the present study we detected a significant greater number of larvae in $STAT6^{+/+}$ compared to $STAT6^{-/-}$ mice at day 5 after infection, but later both groups displayed comparable parasite burdens at 14 and 60 days postinfection (Figure 1(a)). In contrast, in the liver as early as 5 days pi $STAT6^{-/-}$ displayed lower parasite burdens, and this was more evident after 60 days pi (Figure 1(b)). In a similar way the parasite burdens in the brain were statistically different 60 days pi (Figure 1(c)). Interestingly, as infection progressed, the number of larvae in the muscles increased significantly in $STAT6^{+/+}$ mice as compared to $STAT6^{-/-}$ mice that successfully reduced the number of parasites by day 60 postinfection (Figure 1(d); $^*P < 0.01$). Unexpectedly, the lungs from $STAT6^{-/-}$ mice displayed a greater macroscopic damage as we observed an increased number of hemorrhagic spots in such organs (Figure 2(a)) although that numbers of parasites detected in both groups were closely similar at 14 days pi or even lower in $STAT6^{-/-}$ mice at 5 days pi. It is known that *T. canis* infection promotes the recruitment of leukocytes to the lungs generating an acute inflammatory response; here we observed a greater inflammatory infiltration in the lungs of $STAT6^{-/-}$ mice as early as 5 dpi which was maintained higher in these mice until 60 dpi (Figure 2(b)). Moreover, disruption of alveoli was more frequently observed in $STAT6^{-/-}$ mice, as well as a dominant polymorphic cell infiltration (Figure 2(c)). These findings suggest that STAT6-mediated signaling pathway is involved in both susceptibility and pathogenesis during *T. canis* infection in susceptible BALB/c mice.

Previous studies have demonstrated that STAT6-mediated signaling pathway prevents development of protective immunity mainly against intracellular parasites by inhibiting Th1 development [25, 26]. Therefore, we measured levels of Th1-associated IgG2a as well as Th2-associated IgG1 and Total IgE antibodies in $STAT6^{-/-}$ and $STAT6^{+/+}$ mice at different time points following infection with *T. canis*. Additionally, we also compared the cytokine circulating levels from these same mice. Early in the infection, *T. canis*-infected $STAT6^{+/+}$ and $STAT6^{-/-}$ mice displayed minimum and comparable levels of *T. canis* Ag-specific Th1-associated IgG2a antibodies (Figure 3(a)); these data are in line with those previously reported [27], who also found a low production of this subclass of antibody at early times postinfection. However, by day 60 pi $STAT6^{-/-}$ mice displayed significantly higher levels of specific IgG2a antibodies against *Toxocara* antigens (Figure 3(a)). On the other hand, clear differences were observed with the Th2-associated IgG1 production, where $STAT6^{+/+}$ mice displayed significantly higher titers of *Toxocara*-specific IgG1 as compared to $STAT6^{-/-}$ mice since day 14 after infection (Figure 3(b)). Although Th2-associated IgE has been shown to play a role in mediating immunity against certain extraintestinal helminthes, we found that *T. canis*-infected $STAT6^{-/-}$ mice harbored lower parasite burdens despite producing significantly lower levels of IgE as compared to similarly infected $STAT6^{+/+}$ mice, suggesting that IgE may have a limited role in mediating protective immunity against L2 of *T. canis* (Figure 3(c)). Here it is noteworthy that in spite of a higher Th2-associated antibody response in WT mice, these displayed greater susceptibility to *T. canis*. These data agree with those recently reported by [18], who found that after *T. canis* reinfection in BALB/c mice, reinfected mice displayed significantly higher titers of *T. canis*-specific IgG1; moreover, those antibodies showed a greater avidity for *T. canis* antigens. However, re-infected mice displayed a major number of larvae in different tissues [18]. Together with our data, such findings strongly suggest that a humoral immune response is not protective against L2 *T. canis* infection.

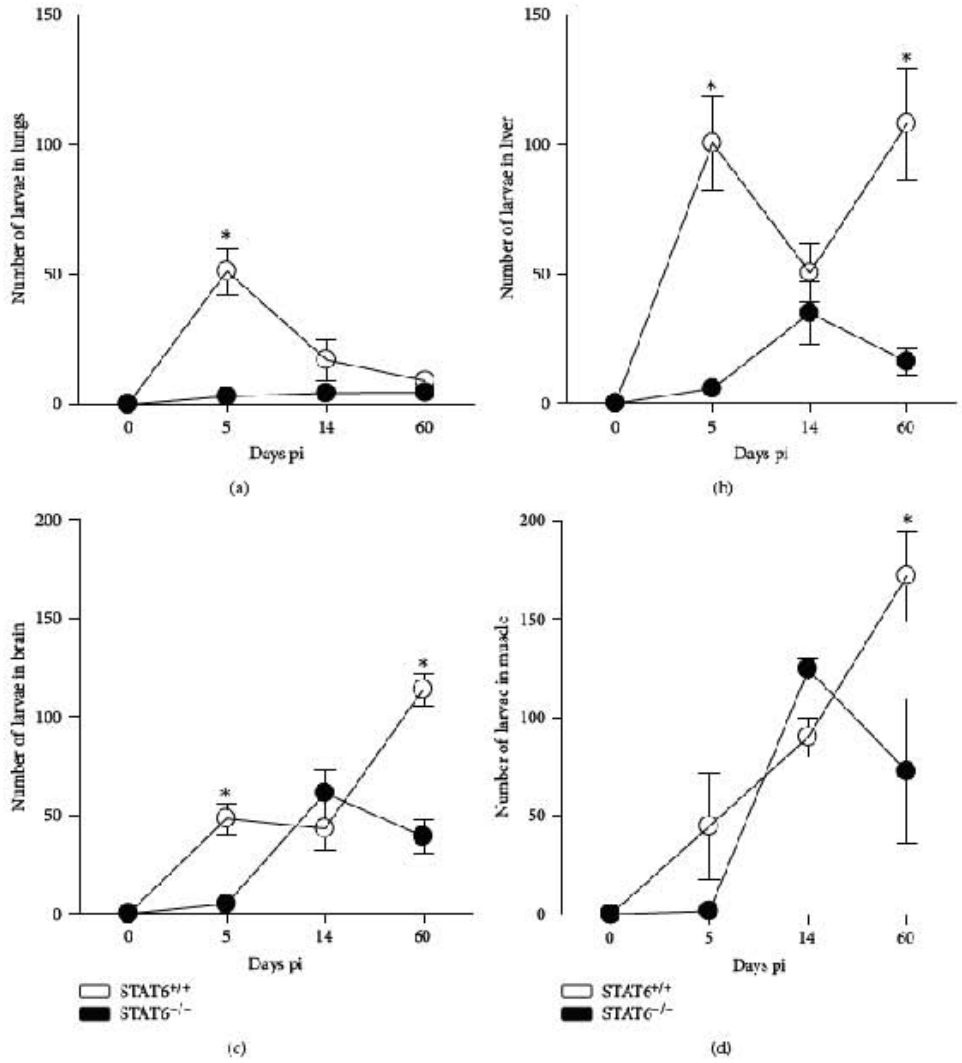

Figure 1: STAT6$^{-/-}$ mice efficiently control *Toxocara canis* infection. Course of i.p. *T. canis* infection in STAT6$^{-/-}$ (solid circles) and STAT6$^{+/+}$ (open circles) mice following infection with 500 L2. Data are expressed as the mean ± SE of 4–6 mice per group. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point. Similar results were observed in two independent experiments.

Next we analyzed the cytokine profile in sera that STAT6$^{+/+}$ and STAT6$^{-/-}$ mice displayed during toxocariasis. BALB/c mice at 5 dpi produced significantly more IL-4 and IFN-γ than STAT6$^{-/-}$ mice (Figures 4(a) and 4(b)), whereas the levels of IL-12 were closely similar between groups (Figure 4(c)), indicating that not a clear Th1-type polarization of the immune response was observed in *T. canis* infected STAT6$^{-/-}$ mice. However, a lack of Th2-type response was confirmed in such mice given the low levels of IgG1 and IgE together with lower systemic levels of IL-4.

To further analyze the immune response, the spleen cells from *T. canis*-infected STAT6$^{+/+}$ and STAT6$^{-/-}$ mice were obtained for RT-PCR analysis of several markers for alternatively activated macrophages (AAM) and some cytokines. While splenocytes from STAT6$^{+/+}$ mice displayed expression of Arginase-1 and Ym-1, both markers for AAM, during early phase of infection, those from STAT6$^{-/-}$ mice did not overexpress such markers (Figure 5(a)). In contrast STAT6$^{-/-}$-infected mice displayed expression of iNOS, a marker for classically activated macrophages, at 14 dpi, whereas STAT6$^{+/+}$

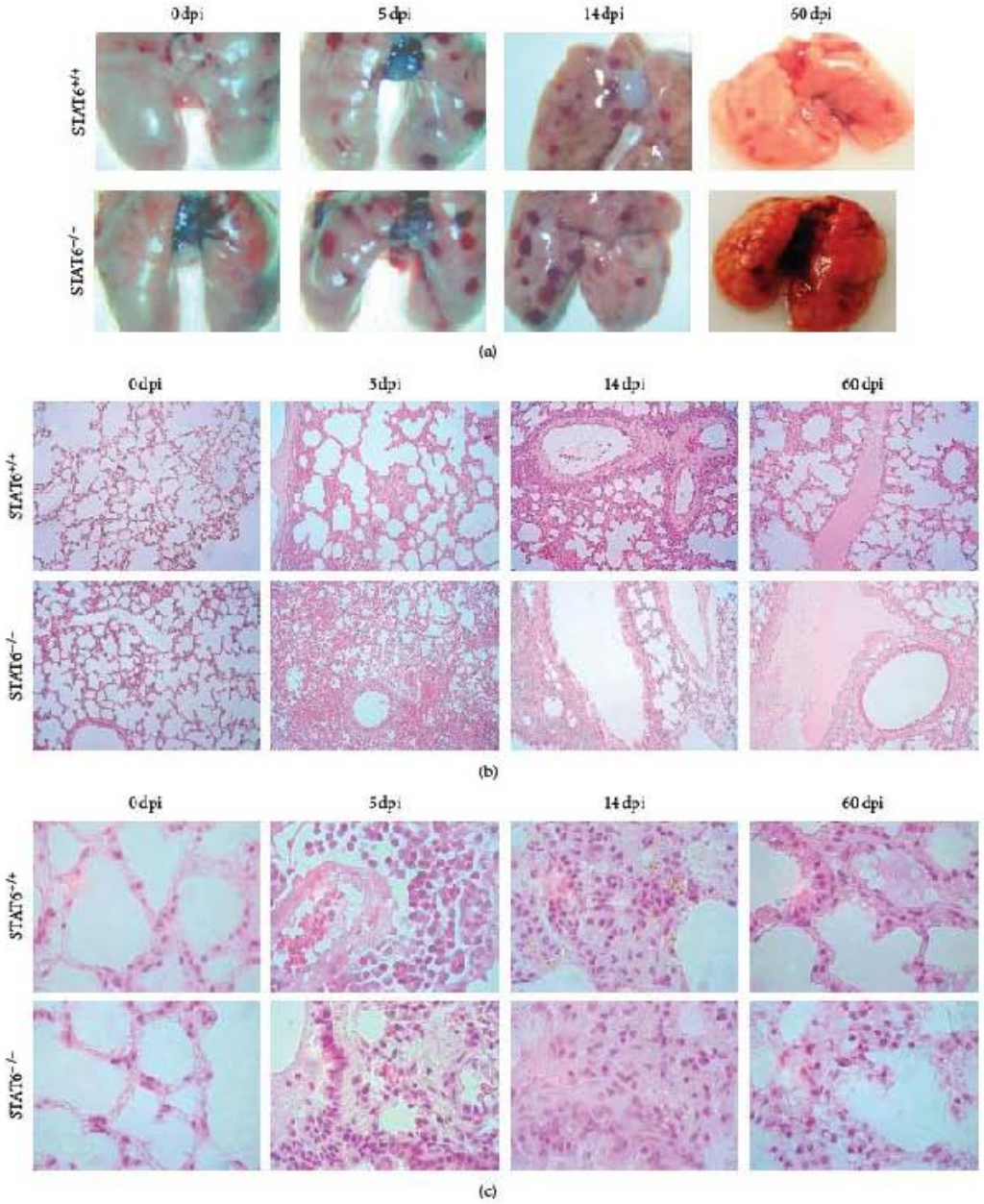

FIGURE 2: STAT6$^{-/-}$ mice display a more severe pathology early in the infection with *T. canis*. (a) Macroscopic appearance of lungs obtained at different time points after oral infection with 500 Larvae of *T. canis*. (b) Lung histology showing airway inflammation in both groups. Magnification 40X. (c) Lung histology. 100X magnification.

mice did not express iNOS (Figure 5(b)). Regarding cytokines we observed a major expression of IL-4 on spleen cells from STAT6$^{+/+}$ than spleen cells from STAT6$^{-/-}$ mice (Figure 5(a)), whereas a similar level of expression was observed for IFN-γ (Figure 5). Thus our data revealed the presence of AAM and IL-4 in the spleens of STAT6$^{+/+}$ mice.

In the last few years, a new cell population has been detected in most of the helminth infections, such population

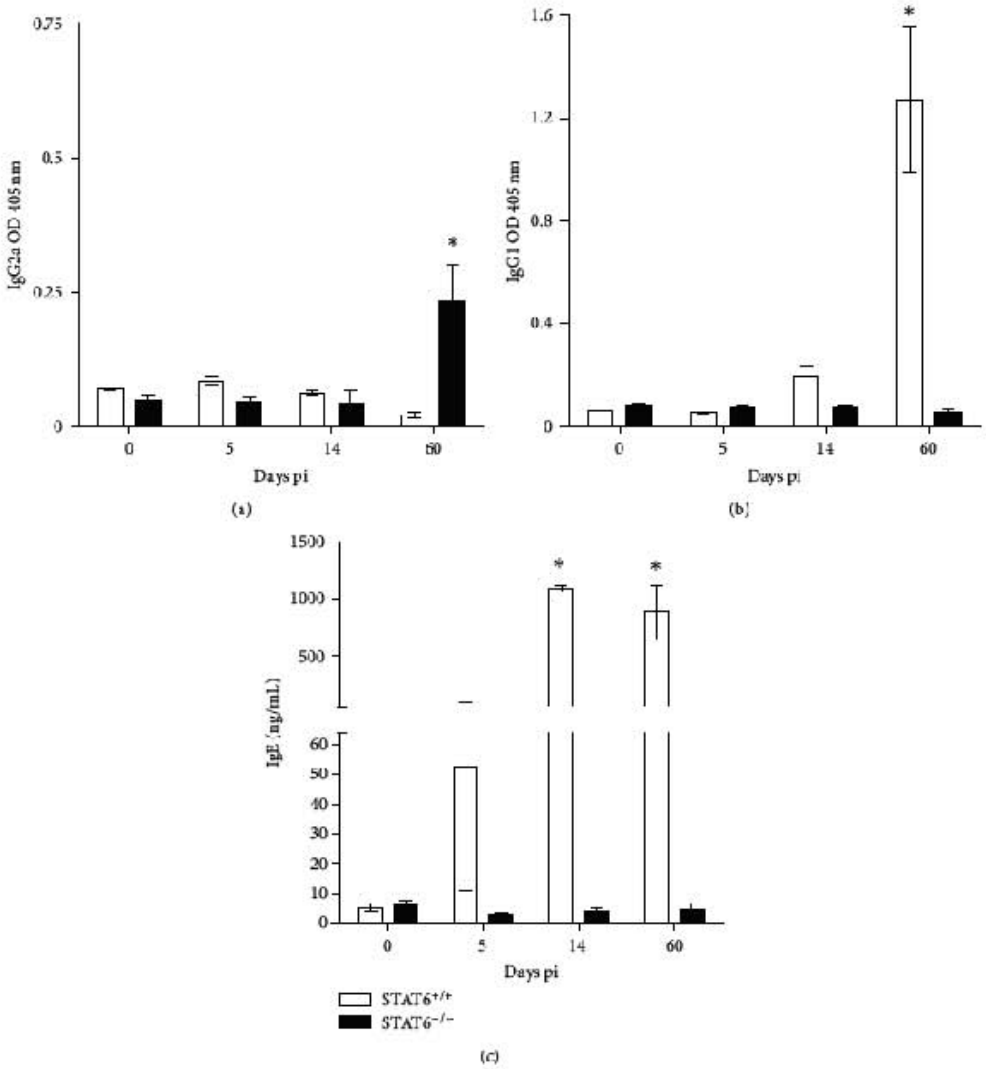

FIGURE 3: Kinetics of antibody production during *T. canis* infection by STAT6$^{-/-}$ and STAT6$^{+/+}$ mice. (a) Anti-*T. canis* specific IgG2a. (b) Anti-*T. canis* specific IgG1 and (c) Total IgE. Sera were taken from the vein tail of each mouse at time points described. ELISA plates were sensitized with 1 μg/well of soluble extract of *T. canis*. The graphs show the mean ± SE (n = 4–6 animals) and are representative of two independent experiments. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point.

is AAM; the role of these cells appears to be divergent [30] as different authors have demonstrated, for example in gastrointestinal infections by *Nippostrongylus brasiliensis* and *Heligmosomoides bakeri* the presence of AAM is key for worm expulsion [31], whereas in other helminth infections such as *Trichuris muris* and *Hymenolepis diminuta* the presence of AAM was irrelevant [32–34]. In contrast, for schistosomiasis and experimental cysticercosis the presence of AAM appears to be crucial; in the first case, the absence of AAM leads to pathologic disorders in the liver and the hosts die [35, 36], whereas in experimental cysticercosis the presence of AAM leads to susceptibility; given that eliminating AAM with clodronate liposomes helps to clear the infection in otherwise susceptible hosts [37]. According to our knowledge this is the first time that the markers for AAM are reported in *T. canis* infection, but their role is still unknown.

In order to gain knowledge on a possible role for AAM in acute *T. canis* infection, we analyzed the profile of

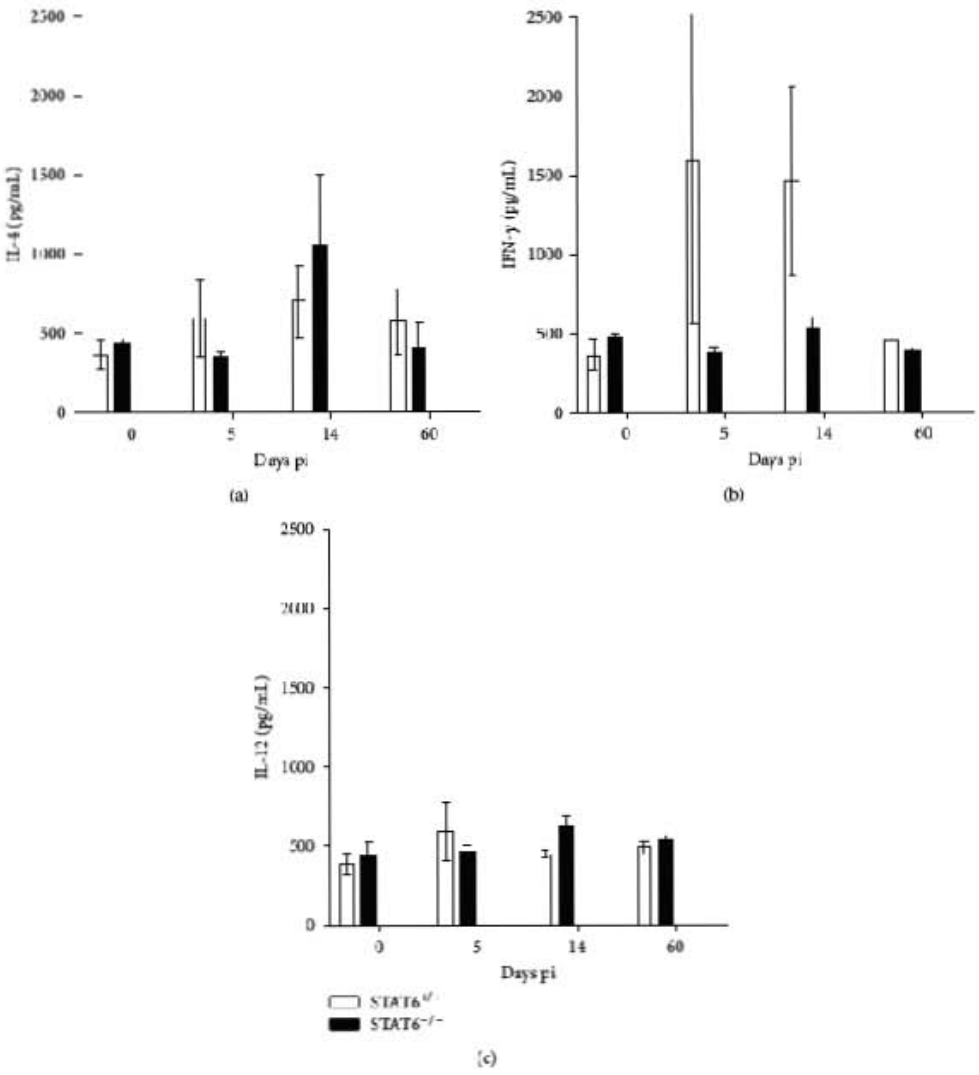

FIGURE 1: Cytokine profiles from the sera of STAT6$^{+/+}$ and STAT6$^{-/-}$ *T. canis*-infected mice. (a) IL-4 detection. (b) IFN-γ production and (c) IL-12 detection. Data are expressed as the mean ± SE and are representative of three independent experiments, $n = 4$. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point.

macrophages that reach the lungs as early as 5 days pi (time where striking differences in pathology were observed). After 5 dpi lungs were obtained and cut in small pieces, which were further passed through a mesh. Cells were stained for different markers and assayed for cytometry. Stained cells were captured in log, and the region that displayed both high granularity and high size was selected for analysis. As observed in Figure 6(a), lung cells from *T. canis*-infected STAT6$^{+/+}$ mice displayed a greater recruitment of F4/80^{+}MR^{+} cells compared to both naïve mice or *T. canis*-infected STAT6$^{-/-}$ mice; these data suggest a recruitment of AAM, which may participate in tissue repair in the lungs, while in STAT6$^{-/-}$ mice the increase of F4/80^{+}MR^{+} cells was gradual, perhaps because they are unable to mount an efficient Th2 response that may impair the recruitment of AAM in the first days pi, which could be associated with a greatest tissue damage

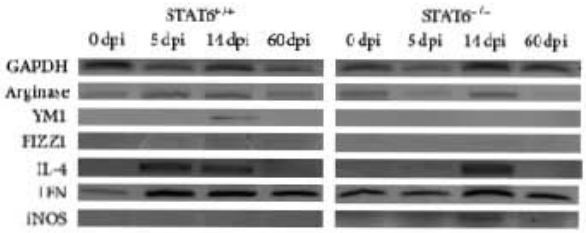

Figure 5: Spleen cells from STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. canis* infected mice display different levels of transcripts. Spleen cells were harvested at different times after infection and transcript levels of GAPDH, Arginase 1, Ym1, Fizz1, IL-4, IFN-γ, and iNOS were analyzed by RT-PCR. The data shown are from a single mouse and are representative of the findings from three mice examined at each time point.

in the lungs (Figure 2(a)). Interestingly, by day 60 pi this population is increased in both groups, but still more damage prevailed in STAT6$^{-/-}$ mice; this apparent contradiction may be explained just in a time-dependent point of view; it means that AAM early recruited in WT mice had more time to repair the tissue. In contrast, the apparent delay of AAM in reaching the lungs in STAT6$^{-/-}$ mice may need longer time to accomplish their function. An alternate explanation may be the participation of different cell populations for a rapid tissue repair, thus, our observation that another population of cells F4/80^{+}-Gr-1^{-} is also recruited differentially in the lungs of STAT6$^{+/+}$ mice may be indicative that more than one-cell population is involved in tissue repair in this infection (Figure 6(b)). Intriguingly, such F4/80^{+} Gr 1^{+} took longer to reach the lungs in STAT6$^{-/-}$ mice. However, these cells reach similar levels than in wild-type mice until 60 dpi (Figure 6(b)), if these cells represent a different population of regulatory cells needs further research. On the other hand, the same pattern of recruitment was followed when we analyzed the F4/80^{+}PDL-1^{+} population (Figure 6(c)). All these surface markers have been previously reported associated with macrophages that undergo a distinct activation phenotype in the presence of the Th2 cytokines or helminth infections named AAM [38]. Besides, these AAM have an upregulated expression of arginase-1, RELM-α, and chitinase-like protein Ym1, among other markers. Moreover, although AAM can exhibit antiparasitic activity [31], their most important function in the context of migrating helminth parasites appears to be associated with tissue-repair responses [39, 40]. Taken together these cytometry analyses with those obtained in the macroscopic and microscopic lung analyses, we may associate less tissue damage on *T. canis*-infected STAT6$^{+/+}$ mice with the presence of AAM, and by contrary, we would associate the absence of AAM in *T. canis*-infected STAT6$^{-/-}$ mice with greater lesions and increased lung cell infiltration. Therefore, we hypothesized that AAM may be involved in mediating protection against helminth-induced immunopathology in the lungs during acute toxocariasis.

Several different reports have shown that Th2 responses are not definitive to kill extraintestinal phases of helminth parasites. For example during muscle infection with *T. spiralis*, BALB/c mice lacking eosinophils displayed similar larval burdens to those of wild-type BALB/c mice [21]. In line with our results, also *T. crassiceps* infection is fully controlled in the absence of STAT6 [22], and more interestingly during experimental neurocysticercosis caused by *Mesocestoides corti* infection STAT6$^{-/-}$ mice displayed a reduction in the number of brain larvae but an increase in clinic neurological symptoms that were associated with lack of AAM [41]. Together all these findings oppose the dogma that Th2-type responses play a critical role in the elimination of all kind of helminthes [42]. These data also suggest that STAT6 pathway may act to limit *Toxocara* larvae-induced immunopathology at least in the lungs.

In conclusion, STAT6$^{-/-}$ BALB/c mice mount a null Th2-like response and efficiently control *T. canis* infection. In contrast, STAT6$^{+/+}$ BALB/c mice develop a predominant Th2-like response that is associated with high levels of IL-4, IgG1, IgE, and AAM and displayed significantly higher parasite burdens in different tissues but interestingly less associated pathology. The findings in our study support the conclusion that STAT6-mediated signaling is critical for the suppression of the immune response that is required for controlling L2 toxocariasis. We postulate that Th2 cytokines may have a dual role during toxocariasis, on one hand may contribute to host susceptibility via STAT6 activation and that neither AAM nor IgE are essential or primarily responsible for eliminating *T. canis* tissue infection, but on the other hand, such response may downregulate the immunopathology induced by *T. canis* larvae in the lungs.

Acknowledgments

This work was supported by PAPIIT-UNAM Grant IN213512, CONACYT Grant 167799, and PAPCA FES-Iztacala 2011-2012 Grant 19. The authors thank MVZ Leticia

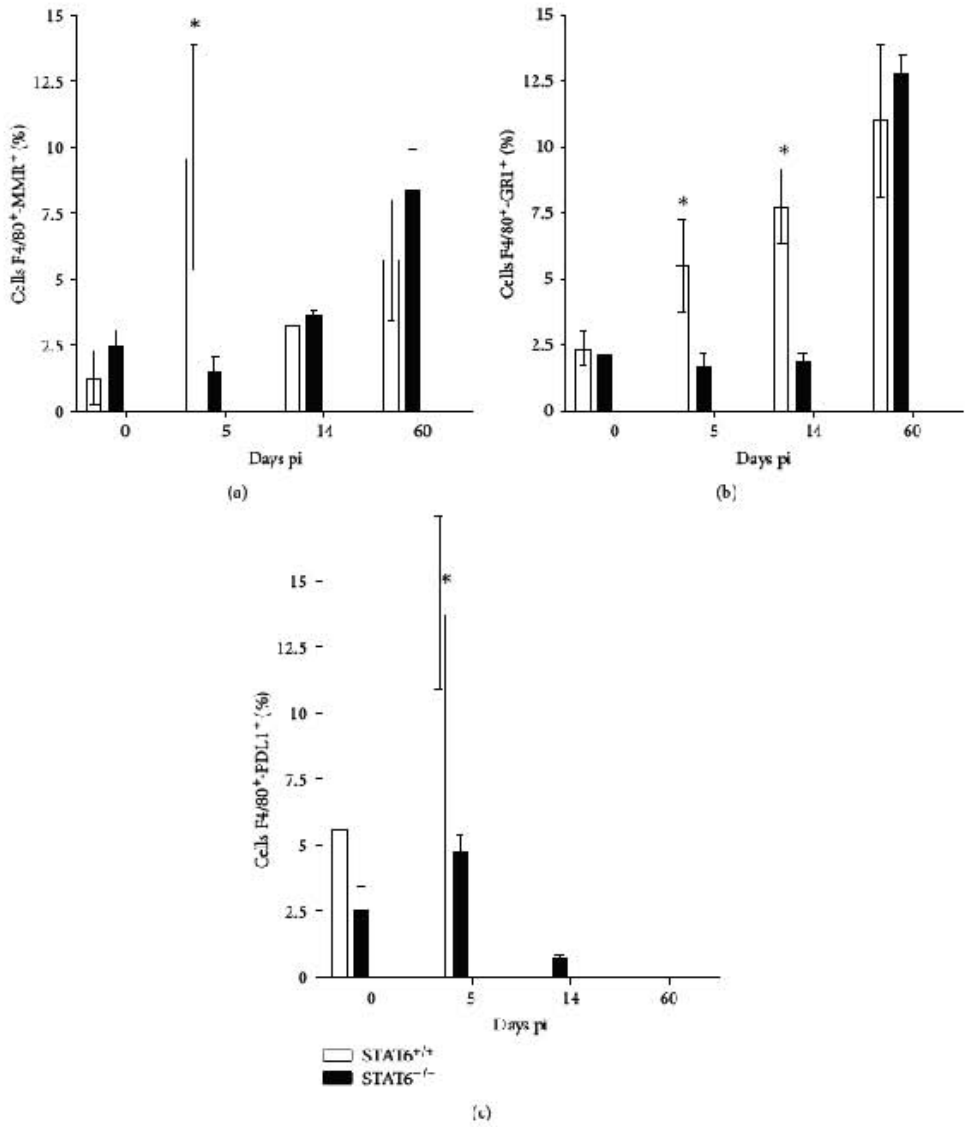

FIGURE 6: Lung macrophages from STAT6$^{-/-}$ and STAT6$^{+/+}$ *T. canis*-infected mice display different phenotypes. Macrophages were obtained at different time points after infection and analyzed by flow cytometry for the detection of different cell markers associated with alternative activation of macrophages. (a) F4/80^{+}MR^{+} lung cells, (b) F4/80^{+}Gr1^{+} lung cells, and (c) F4/80^{+}PD-L1^{+} lung cells, as described in Section 2. Data are expressed as the mean ± SE and are representative of two independent experiments, $n = 4$. $^{*}P < 0.05$ comparing STAT6$^{-/-}$ versus STAT6$^{+/+}$ at the same time point. ND: not determined.

Flores and Tomas Villamar for their excellent care of animals and Biol. Ana F. Chavez Sanchez for histology assistance.

References

[1] H. Avcioglu and I. Balkaya, "The relationship of public park accessibility to dogs to the presence of Toxocara species ova in the soil," *Vector-Borne and Zoonotic Diseases*, vol. 11, no. 2, pp. 177–180, 2011.

[2] V. C. C. Dattoli, S. M. Freire, L. R. Mendonça, P. C. Santos, R. Meyer, and N. M. Alcantara-Neves, "*Toxocara canis* infection is associated with eosinophilia and total IgE in blood donors from a large Brazilian centre," *Tropical Medicine and International Health*, vol. 16, no. 4, pp. 514–517, 2011.

[3] P. Congdon and P. Lloyd, "Toxocara infection in the United States: the relevance of poverty, geography and demography as riskfactors, and implications for estimating county prevalence," *International Journal of Public Health*, vol. 56, no. 1, pp. 15–24, 2011.

[4] F. Pinelli, T. Herremans, M. G. Harms, D. Hoek, and L. M. Kortbeek, "Toxocara and Ascaris seropositivity among patients suspected of visceral and ocular larva migrans in the Netherlands: trends from 1998 to 2009," *European Journal of Clinical Microbiology and Infectious Diseases*, pp. 1–7, 2011.

[5] M. G. Walsh, "Toxocara infection and diminished lung function in a nationally representative sample from the United States population," *International Journal for Parasitology*, vol. 41, no. 2, pp. 243–247, 2011.

[6] H. L. D. C. Amaral, G. L. Rassier, M. S. Pepe et al., "Presence of *Toxocara canis* eggs on the hair of dogs: a risk factor for Visceral Larva Migrans," *Veterinary Parasitology*, vol. 174, no. 1-2, pp. 115–118, 2010.

[7] W. F. El Tras, H. R. Holt, and A. A. Tayel, "Risk of *Toxocara canis* eggs in stray and domestic dog hair in Egypt," *Veterinary Parasitology*, vol. 178, no. 3-4, pp. 319–323, 2011.

[8] R. A. Jabbour, S. S. Kanj, R. A. Sawaya, G. N. Awar, M. H. Hourani, and S. F. Atweh, "*Toxocara canis* myelitis: clinical features, magnetic resonance imaging (MRI) findings, and treatment outcome in 17 patients," *Medicine*, vol. 90, no. 5, pp. 337–343, 2011.

[9] C. M. Hamilton, S. Brandes, C. V. Holland, and E. Pinelli, "Cytokine expression in the brains of *Toxocara canis*-infected mice," *Parasite Immunology*, vol. 30, no. 3, pp. 181–185, 2008.

[10] F. D. Finkelman, T. Shea-Donohue, J. Goldhill et al., "Cytokine regulation of host defense against parasitic gastrointestinal nematodes: lessons from studies with rodent models," *Annual Review of Immunology*, vol. 15, pp. 505–533, 1997.

[11] W. C. Gause, T. F. Urban Jr, and M. J. Stadecker, "The immune response to parasitic helminths: insights from murine models," *Trends in Immunology*, vol. 24, no. 5, pp. 269–277, 2003.

[12] M. Morimoto, A. Zhao, R. Sun et al., "IL 13 receptor α2 regulates the immune and functional response to Nippostrongylus brasiliensis infection," *Journal of Immunology*, vol. 183, no. 3, pp. 1934–1939, 2009.

[13] J. F. Urban Jr, C. R. Maliszewski, K. B. Madden, I. M. Katona, and F. D. Finkelman, "IL-4 treatment can cure established gastrointestinal nematode infections in immunocompetent and immunodeficient mice," *Journal of Immunology*, vol. 154, no. 9, pp. 4675–4684, 1995.

[14] J. G. Perrigoue, F. A. Marshall, and D. Artis, "On the hunt for helminths: innate immune cells in the recognition and response to helminth parasites," *Cellular Microbiology*, vol. 10, no. 9, pp. 1757–1764, 2008.

[15] M. L. Knott, K. J. Matthaei, P. S. Foster, and L. A. Dent, "The roles of eotaxin and the STAT6 signalling pathway in eosinophil recruitment and host resistance to the nematodes Nippostrongylus brasiliensis and Heligmosomoides bakeri," *Molecular Immunology*, vol. 45, no. 13, pp. 2714–2722, 2009.

[16] F. D. Finkelman, T. Shea-Donohue, S. C. Morris et al., "Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites," *Immunological Reviews*, vol. 201, pp. 139–155, 2004.

[17] J. F. Urban Jr, N. Noben-Trauth, D. D. Donaldson et al., "IL-13, IL-4Rα, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis," *Immunity*, vol. 8, no. 2, pp. 255–264, 1998.

[18] P. Kolbeková, D. Větvička, J. Svoboda et al., "*Toxocara canis* larvae reinfecting BALB/c mice exhibit accelerated speed of migration to the host CNS," *Parasitology Research*, vol. 109, no. 5, pp. 1267–1278, 2011.

[19] A. A. Othman, S. H. El-Shourbagy, and R. H. Soliman, "Kinetics of Foxp3-expressing regulatory cells in experimental *Toxocara canis* infection," *Experimental Parasitology*, vol. 127, no. 2, pp. 454–459, 2011.

[20] L. I. Terrazas, R. Bojalil, T. Govezensky, and C. Larralde, "Shift from an early protective TH1-type immune response to a late permissive TH2-type response in murine cysticercosis (*Taenia crassiceps*)," *Journal of Parasitology*, vol. 84, no. 1, pp. 74–81, 1998.

[21] V. Fabre, D. P. Beiting, S. K. Bliss et al., "Eosinophil deficiency compromises parasite survival in chronic nematode infection," *Journal of Immunology*, vol. 182, no. 3, pp. 1577–1583, 2009.

[22] M. Rodríguez-Sosa, I. R. David, R. Bojalil, A. R. Satoskar, and L. I. Terrazas, "Cutting edge: susceptibility to the larval stage of the helminth parasite *Taenia crassiceps* is mediated by Th2 response induced via STAT6 signaling," *Journal of Immunology*, vol. 168, no. 7, pp. 3135–3139, 2002.

[23] H. Takeda, T. Tanaka, W. Shi et al., "Essential role of Stat6 in IL-4 signalling," *Nature*, vol. 380, no. 6575, pp. 627–630, 1996.

[24] J. F. Urban Jr, L. Schopf, S. C. Morris et al., "Stat6 signaling promotes protective immunity against Trichinella spiralis through a mast cell- and T cell-dependent mechanism," *Journal of Immunology*, vol. 164, no. 4, pp. 2046–2052, 2000.

[25] L. M. Stamm, A. Raisanen-Sokolowski, M. Okano, M. E. Russell, J. E. David, and A. R. Satoskar, "Mice with STAT6-targeted gene disruption develop a Th1 response and control cutaneous leishmaniasis," *Journal of Immunology*, vol. 161, no. 11, pp. 6180–6188, 1998.

[26] R. L. Tarleton, M. J. Grusby, and L. Zhang, "Increased susceptibility of stat4-deficient and enhanced resistance in stat6-deficient mice to infection with Trypanosoma cruzi," *Journal of Immunology*, vol. 165, no. 3, pp. 1520–1525, 2000.

[27] S. Fenoy, M. Rodero, E. Pons, C. Aguila, and C. Cuellar, "Follow-up of antibody avidity in BALB/c mice infected with *Toxocara canis*," *Parasitology*, vol. 135, no. 6, pp. 725–733, 2008.

[28] J. L. Reyes, A. F. Espinoza-Jiménez, M. I. González, L. Verdin, and L. I. Terrazas, "*Taenia crassiceps* infection abrogates experimental autoimmune encephalomyelitis," *Cellular Immunology*, vol. 267, no. 2, pp. 77–87, 2011.

[29] W. I. Khan, E. A. Vallance, P. A. Blennerhassett et al., "Critical role for signal transducer and activator of transcription factor 6 in mediating intestinal muscle hypercontractility and worm expulsion in Trichinella spiralis-infected mice," *Infection and Immunity*, vol. 69, no. 2, pp. 838–844, 2001.

[30] D. M. McKay and W. I. Khan, "STAT-6 is an absolute requirement for murine rejection of Hymenolepis diminuta," *Journal of Parasitology*, vol. 89, no. 1, pp. 188–189, 2003.

[31] R. M. Anthony, L. F. Urban, F. Alem et al., "Memory TH2 cells induce alternatively activated macrophages to mediate protection against nematode parasites," *Nature Medicine*, vol. 12, no. 8, pp. 955–960, 2006.

[32] R. Rinaldi, T. V. Bell, M. Little et al., "Arginase-1-expressing macrophages are dispensable for resistance to infection with the gastrointestinal helminth Trichuris muris," *Parasite Immunology*, vol. 33, no. 7, pp. 411–420, 2011.

[33] M. Becerra-Díaz, H. Valderrama-Carvajal, and L. I. Terrazas, "Signal transducers and activators of transcription (STAT)

family members in helminth infections," *International Journal of Biological Sciences*, vol. 7, no. 9, pp. 1371–1381, 2011.

[34] M. J. G. Johnston, A. Wang, M. E. D. Catarino et al., "Extracts of the rat tapeworm, Hymenolepis diminuta, suppress macrophage activation in vitro and alleviate chemically induced colitis in mice," *Infection and Immunity*, vol. 78, no. 3, pp. 1364–1375, 2010.

[35] D. R. Herbert, T. Orekov, A. Roloson et al., "Arginase I suppresses IL-12/IL-23p40-driven intestinal inflammation during acute schistosomiasis," *Journal of Immunology*, vol. 184, no. 11, pp. 6438–6446, 2010.

[36] D. R. Herbert, C. Hölscher, M. Mohrs et al., "Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology," *Immunity*, vol. 20, no. 5, pp. 623–635, 2004.

[37] J. L. Reyes, C. A. Terrazas, J. Alonso-Trujillo, N. van Rooijen, A. R. Satoskar, and L. I. Terrazas, "Early removal of alternatively activated macrophages leads to *Taenia crassiceps* cysticercosis clearance in vivo," *International Journal for Parasitology*, vol. 40, no. 6, pp. 731–742, 2010.

[38] J. L. Reyes and L. I. Terrazas, "The divergent roles of alternatively activated macrophages in helminthic infections," *Parasite Immunology*, vol. 29, no. 12, pp. 609–619, 2007.

[39] J. E. Allen and R. M. Maizels, "Diversity and dialogue in immunity to helminths," *Nature Reviews Immunology*, vol. 11, no. 6, pp. 375–388, 2011.

[40] S. J. Jenkins and J. E. Allen, "Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes," *Journal of Biomedicine & Biotechnology*, vol. 2010, Article ID 262609, 14 pages, 2010.

[41] B. B. Mishra, U. M. Gundra, and J. M. Teale, "STAT6$^{-/-}$ mice exhibit decreased cells with alternatively activated macrophage phenotypes and enhanced disease severity in murine neurocysticercosis," *Journal of Neuroimmunology*, vol. 232, no. 1-2, pp. 26–34, 2011.

[42] R. M. Maizels, J. P. Hewitson, and K. A. Smith, "Susceptibility and immunity to helminth parasites," *Current Opinion in Immunology*, vol. 24, no. 4, pp. 459–466, 2012.