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" *Taenia crassiceps* y sus productos controlan la diabetes experimental tipo 1"

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RESUMEN

La diabetes mellitus tipo 1 (T1DM) es una enfermedad autoinmune que afecta principalmente a niños y jóvenes. Los linfocitos T CD4 y CD8, como también macrófagos clásicamente activados (CAMϕS) destruyen gradualmente las células β pancreáticas, que producen insulina. Cuando la mayoría de las células β son destruidas, la habilidad del páncreas para secretar insulina en respuesta a los niveles de glucosa es deficiente, provocando hiperglucemia crónica asociada a disfunción y falla de varios órganos como ojos, riñones, corazón, vasos sanguíneos. Citocinas inflamatorias como IL-1β, IL-6, IFN-γ, TNF-α, y óxido nítrico (NO) son liberados por las mismas células inmunes autoreactivas y son importantes en el desarrollo de la diabetes. Existen varios modelos experimentales que nos ayudan a resolver los enigmas de la enfermedad, uno de los más utilizados es la inducción de T1DM por inyecciones múltiples de dosis bajas de estreptozotocina (MLD-STZ).

Los helmintos parásitos modulan el sistema inmune de sus hospederos induciendo una fuerte respuesta Th2 y el incremento de poblaciones como linfocitos T reguladores (Treg) y macrófagos alternativamente activados (AAMϕS) y disminuyen los efectos inflamatorios. Se ha postulado que, algunas infecciones por helmintos pueden prevenir o retrasar el desarrollo de enfermedades autoinmunes como la T1DM. Previamente, comprobamos que, ratones inducidos con T1DM por medio de MLD-STZ e infectados con *Taenia crassiceps* disminuyen significativamente la hiperglucemia, insulitis y la incidencia de la T1DM. Además, encontramos altos títulos de IL-4 en suero y un incremento significativo de la población de AAMϕS, que sugieren que esta población puede ser importante en la protección contra la T1DM.

El presente estudio tiene como finalidad comprobar sí el antígeno soluble (TcS) o el excretado-secretado (TcES) derivados de *T. crassiceps* puede proteger de la T1DM. Analizamos diferentes dosis y elegimos el mejor tratamiento, antes o post-inducción de la T1DM. Examinamos glucemia en sangre, incidencia, insulitis, perfil de citocinas y macrófagos peritoneales. Nuestros datos

demuestran que el tratamiento con el TcS protege de la T1DM en ratones, siempre y cuando, el tratamiento se administre antes de la inducción de diabetes y de manera constante. Por otra parte, el tratamiento con TcES puede reducir la T1DM incluso si se inicia una semana después de la inducción de la T1DM. En ambos casos, se incrementan las poblaciones de AAMϕs y CD11b+Gr1+ en peritoneo. Finalmente, examinamos el papel de los AAMϕS modulando la T1DM en ratones infectados con *T. crassiceps*, en los cuales se ha eliminado esta población con liposomas con clodronato.

En conclusión, nuestros datos demuestran que la administración de antígenos de *T. crassiceps* pueden reducir y controlar de forma importante la T1DM. El tratamiento con inyecciones de TcES fue considerado mejor que el TcS ya que pudo reducir la hiperglucemia, incidencia e insulitis de forma efectiva incluso cuando el tratamiento inició después de la inducción de T1DM. Además, con este trabajo se comprueba que los AAMϕS cumplen una función importante como células responsables de la modulación de la diabetes, y su ausencia genera fuertes signos de la enfermedad.

ABSTRACT

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that affects mostly children and young people less 20 years. CD4+ and CD8+ lymphocytes, as well classically activated macrophages (CAMϕS) are involved in the selective destruction of insulin-producing β cells. When mostly of β-cells are destroyed, pancreas ability to secrete insulin in response to blood glucose levels is impaired, resulting in chronic hyperglycemia related to dysfunction and failure to many organs such eyes, kidneys, hearth, blood vessels. Proinflammatory cytokines such IL-1β, IL-6, TNF-α, IFN-γ and NO are release by autoreactive cells and which seem to be important to development of T1DM. There are so many options of experimental models for understand T1DM, the MLD-STZ is one of useful tool to solve it.

Helminths infections can modulate immune system of its host, they induce a strong Th2-type response and increase cell populations, such T cells regulatory (Treg) and alternatively activated macrophages (AAMϕS) and reducing inflammatory response. Interestingly, in many studies, some helminth infections could reduce or delay development of chronic autoimmne disease as T1DM. Previously, we have shown that *Taenia crassiceps* infected mice and treated with MLD-STZ, significantly decreased hyperglycemia, insulitis and consequently the incidence of T1DM. Moreover, higher titles of IL-4 in sera with increase of AAMϕS population, suggest that this population could be important in protection against T1DM.

The purpose of this study was to determine if soluble (TcS) or excreted/secreted (TcES) antigen of *T. crassiceps* could protect against T1DM induced by MLD-STZ. Also, we analyzed different doses and we chose which were the best treatment before or post-induction of T1DM in MLD-STZ mice. We also tested blood glucose, incidence, insulitis, cytokines profiles and peritoneal macrophages. Our data shown that TcS antigen protect if treatment was constant and before induction of T1DM in MLD-STZ mice; in contrast, TcES antigen was effective to reduce incidence of T1DM with a higher doses (200 µg) in a post-induction MLD-STZ treatment. In both cases, the presence of AAMϕS and $GR1^{+}CD11b^{+}$ populations were increased. Finally, we examined paper of

AAMϕS modulating T1DM in mice infected with *T. crassiceps* which were deleted mostly of these population with injection of clodronate-loaded liposomes.

In conclusion, our data demonstrate that injection of *T. crassiceps* antigens could reduce and control T1DM. Treatment of TcES was considered better than TcS injections in mice because was effective decrease hyperglycemia, incidence and insulitis even when TcES treatment was starting post-induction of MLD-STZ treatment. On the future, TcES maybe can be considered as a treatment against T1DM. Furthermore, in this study we demonstrated that AAMϕS were important cells maintains immune-regulation of T1DM and their absence generates serious signs of T1DM.

ABREVIACIONES

AAMϕS	Macrófagos Alternativamente Activados
APC	Aloficocianina
CAMϕS	Macrófagos Clásicamente Activados
CCR2	Receptor de quimicinas tipo 2
ELISA	Ensayo de inmunoabsorción ligado a enzimas
FITC	Fluoresceina
IFN-γ	Interferon-gamma
IL	Interleucina
iNOS	Oxido nítrico sintasa
i.p.	Intraperitoneal
Jak	Tirosina-cinasas
p.i.	posterior a la inducción de la T1D
MCP-1	Citocina quimiotáctica de monocitos-1
MDSCs	Células Mieloides supresoras
MLD-STZ	Dosis bajas de estreptozotocina (Multiple Low doses of streptozotocine)
NF-kB	Factor nuclear kB
NOD	Ratones diabéticos no-obesos
PE	Ficoeritrina
PECS	Células obtenidas del peritoneo de los ratones
PBS	Amortiguador de fosfatos
ROS	Especies reactivas de oxigeno
STZ	Estreptozotocina
TcES	Antígeno de *T. crassiceps* de productos excretados/secretados
TcS	Antígeno soluble de *T. crassiceps*
TNF-α	Factor de necrosis tumoral α

Treg	Células T reguladoras
T1DM	Diabetes mellitus tipo 1
Untreated	Ratones sin tratamiento

INTRODUCCIÓN

Diabetes Mellitus tipo 1

Es una enfermedad autoinmune que afecta del 5-10% de la población mundial, teniendo una mayor incidencia en niños y adolescentes [1]. El desarrollo de dicha enfermedad se debe al ataque agresivo de células inmunes autoreactivas que reconocen antígenos de las células β como extraños y las eliminan. La enfermedad sigue dos etapas principales: la insulitis, en la cual, células del sistema inmune invaden los islotes pancreáticos, promoviendo la muerte de las células β, productoras de insulina; y la diabetes, se considera la fase donde existe una pérdida importante de células β y el páncreas no puede producir la suficiente insulina para controlar los niveles de glucosa en sangre, por tanto, se presenta hiperglucemia crónica que conduce al daño en diferentes órganos, como corazón, ojos, vasos sanguíneos. Las personas con T1DM son diagnosticadas tardíamente y por tanto son pacientes dependientes de insulina. Hasta el momento no existe algún tratamiento que pueda curar efectivamente de la T1DM [1, 2].

Los CAMϕS, linfocitos Th1 y T CD8+ autoreactivos son las primeras células en infiltrar los islotes pancreáticos [3]. Las citocinas liberadas por dichas células como TNF-α, IL-1β e IL-6 así como radicales libres se incrementan durante la inflamación y activan distintas vías de señalización. IL-1β y TNF-α activan al factor de transcripción, Factor nuclear kB (NF-kB), el cual promueve la apoptosis de las células β incrementando la expresión de FAS. Por otra parte, IFN-γ junto con TNF-α, activan las tirosina-cinasas JAK1 y JAK2 activando al factor de transcripción STAT-1 e induciendo la sobreexpresion de iNOS, la secreción de óxido nítrico (NO), promoviendo la apoptosis de las células β vía p-53 [3, 4]. Los radicales libres, a su vez, inducen apoptosis (por medio de la activación de caspasas) e incrementan el ciclo vicioso de inflamación [4]. Durante este proceso, también se liberan quimiocinas como MCP-1 (también conocida como CCL2) la cual es importante en el reclutamiento de CAMϕS, monocitos, células dendríticas y linfocitos T activados vía su receptor CCR2 [5, 6].

De manera experimental existen varios métodos para inducir T1DM. Uno de los más utilizados son los ratones diabéticos no-obesos (non-obese diabetic, NOD), los cuales espontáneamente desarrollan T1DM. Las hembras son más susceptibles que los machos, y comienzan a desarrollar insulitis después de la semana 4-5 de edad. Los signos de la T1DM como hiperglucemia, destrucción masiva de las células β, se manifiestan con mayor claridad de la semana 12 a la 30 [7]. Otro modelo experimental muy utilizado, es por medio de un agente tóxico. La estreptozotocina (STZ) es una toxina derivada de *Streptomyces achromogenes* y se utiliza para inducir diabetes tipo 1 en ratones susceptibles [8]. Es un poderoso agente alquilante que, interfiere con el transporte de glucosa, actúa como productor de ROS e induce ruptura del DNA [9]. La inyección de MLD-STZ en animales susceptibles pueden producir insulitis pancreática con una destrucción progresiva de las células β e hiperglucemia, se ha observado que los macrófagos son células relacionadas con el desarrollo e iniciación de la T1DM en ratones inducidos con MLD-STZ [10].

Macrófagos

Los macrófagos son células derivadas de monocitos, los cuales a su vez se diferencian a partir de precursores mieloides en la médula ósea y están ampliamente distribuidos en todos los tejidos del cuerpo. Estas células son importantes tanto en la respuesta inmune innata como adaptativa, realizando distintas funciones [11]. La enorme capacidad fagocítica de estas células les permite ejercer funciones efectoras dentro de la respuesta inmune, por lo cual se relacionan con la eliminación de patógenos unicelulares [12]. Los macrófagos también tienen la capacidad de presentar antígenos mediante la expresión de MHC-II y de secretar diversas citocinas, con lo que pueden activar a los linfocitos T CD4 e inducir su proliferación hacia perfiles Th1, Th2, Th17 y Treg [12, 13]. Estas células también se han visto implicadas en la eliminación de células apoptóticas y necróticas así como en la regulación del metabolismo de la glucosa y los lípidos, contribuyendo al mantenimiento de la homeostasis [14]. Se han definido principalmente dos poblaciones, los macrófagos clásicamente activados (CAMϕS o M1) y los macrófagos alternativamente activados (AAMϕS o M2) [11, 14, 15].

Los CAMϕS se inducen durante respuestas proinflamatorias, mediante el estímulo de IFN-γ, y de algunos productos bacteriales como el LPS, o bien responden a infecciones de patógenos intracelulares como *Mycobacterium tuberculosis, Toxoplasma gondii, Leishmania major* [13]. Las citocinas que producen son TNF-α, IL-12, IL-1β, IL-23 y especies reactivas de óxigeno (ROS) como el NO producido por la óxido nítrico sintasa (iNOS). Estos macrófagos incrementan su capacidad de presentar antígeno. Los CAMϕS son células dominantes que promueven el desarrollo de la diabetes tipo 1 y 2 [11, 14].

En contraste, los AAMϕS son inducidos durante respuestas de tipo Th2, como las infecciones con helmintos y alergias. Los AAMϕS producen IL-10 y TGF- β, por tanto, la expresión de citocinas proinflamatorias es muy baja o nula. Además, los AAMϕS producen urea, poliaminas y L-ornithina asociado con alta expresión de arginasa-1 (Arg-1), la cual compite con la iNOs por su sustrato común, la L-arginina, de este modo se encuentra implicada en la inhibición de la producción de NO [15, 16]. En los AAMϕs también se incrementa la expresión de Ym1, relacionada con el reclutamiento de eosinofilos, fortaleciendo la polarización de la respuesta inmune hacia un perfil Th2. Los ligandos de PD-1 (Programmed Death-1) PDL1 y PDL2, también se expresan en mayor magnitud, contribuyendo a inducir bajas respuestas proliferativas en los linfocitos T [17]. En algunas infecciones por helmintos como *Brugia malayi, Litomosoides sigmodontis, Nippostrongylus brasiliensis, Heligmosomoides polygyrus, Schistosoma mansoni, Fasciola hepática, Taenia crassiceps, Hymenolepis diminuta* y *Echinococcus multicularis* se inducen respuestas Th2, las cuales son acompañadas por la inducción de poblaciones de AAMϕS [13].

Células Mieloides Supresoras

Las células mieloides supresoras se descubrieron hace 20 años en pacientes con cáncer, pero ahora han cobrado interés, ya que se han relacionado en varias enfermedades e infecciones parasitarias. Las células mieloides supresoras es una población heterogénea que consiste de células mieloides progenitoras y células mieloides inmaduras [18, 19]. En condiciones fisiológicas, las células mieloides inmaduras se diferencian en progenitoras y posteriormente, en células dendríticas, macrófagos, neutrófilos, granulocitos. En contraste, en condiciones patológicas,

como cáncer, parásitos, sepsis, trauma, trasplante de médula ósea, autoinmunidad, las células mieloides inmaduras pueden bloquear su maduración y expandirse como células mieloides supresoras (MDSCs) [20].

Es difícil poder identificar a las MDSCs, ya que expresan muchos marcadores leucocitarios, aunque se han caracterizado algunos, tanto en humanos como en ratones. En ratones, las MDSCs se diferencian por una alta expresión de CD11b y Gr1. Se pueden clasificar dos poblaciones importantes: las células granulocíticas que expresan $CD11b^{+}Ly6G^{+}Ly6C^{low}$ y las células monocíticas, que expresan $CD11b^{+}Ly6G^{-}Ly6C^{hi}$. En humanos, es un poco más complicado su identificación, pero se ha podido observar en la mayoría de los estudios una alta expresión de CD11b y CD33, así como nula expresión de HLA-DR, e inclusive, algunos autores las clasificaron también en dos subpoblaciones, $CD15^{+}$ como la población granulocítica y la población $CD14^{+}$ como monocítica [20-22].

Las MDSCs ejercen inmunosupresión por comunicación cruzada con linfocitos T, NKs, macrófagos, dendríticas, y otras células del sistema inmune, vía contacto celular, MHC-peptido-TCR-CD28, o bien, por mediadores solubles como ON, ROS, IL-10, TGF-β [20]. Además, las MDSCs pueden inhibir la función de las células dendríticas, evitando su migración, toma del antígeno, y cambiando el proceso inflamatorio a uno anti-inflamatorio. Otra función de las MDSCs es con los macrófagos, disminuyendo la inflamación por la disminución en la producción de IL-2, IL-6 y MHC-II. Otro punto importante, es que las MDSCs pueden promover la producción de Treg [21]. En las infecciones por parásitos, las MDSCs pueden aparecer y tener ciertas funciones. Por ejemplo, en la infección aguda con *Trypanosoma cruzi*, la activación de los linfocitos T y la producción de IFN-γ lleva a la expansión de las MDSCs. Esto mismo, se ha reportado en la toxoplasmosis aguda, sepsis, en la infección crónica con *Leishmania major*, entre otros [21, 22].

Se ha demostrado, que las MDSCs pueden ser células reguladoras importantes de la diabetes tipo 1. En un estudio se demostró, que la transferencia de MDSCs, obtenidas de ratones con tumores, y transferidas en ratones diabéticos, incrementaron la población de Treg, disminuyendo la incidencia de la diabetes significativamente comparada con los animales diabéticos a los que no les transfirieron MDSCs [23].

Helmintos

Los helmintos parásitos son un grupo de organismos diverso que presentan diferentes morfologías, estructuras accesorias, tipos de alimentación, tipos de reproducción y etapas del ciclo de vida [24]. Sorprendentemente a pesar de estas diferencias, los helmintos comparten la habilidad de ejercer fuertes efectos reguladores sobre el sistema inmune de sus hospederos [25]. La infección por helmintos induce una potente respuesta Th2 e induce la aparición de poblaciones reguladoras como los AAMϕS o Treg. Numerosas evidencias experimentales de modelos murinos de autoinmunidad y su regulación mediante infecciones por helmintos apoya el papel protector de la respuesta Th2 propuestos en la hipótesis de la higiene [12, 26, 27]**.** Por ejemplo, se ha demostrado que la infección por *H. poligyrus* en ratones NOD tiene un efecto protector en la T1D, disminuyendo la incidencia, el infiltrado celular, las poblaciones celulares como CD4, CD8, macrófagos y células dendríticas implicadas en la insulitis y el daño a las células β en el páncreas. A su vez, se incrementan poblaciones reguladoras como los AAMϕS en los ganglios pancreáticos y periféricos [28]. En la infección con *S. mansoni* o bien la administración de antígeno de huevos de *S.mansoni* (SEA) en ratones NOD, encontraron que hay una relación directa entre menor incidencia e insulitis con el incremento de las poblaciones de AAMϕS y Treg [29-31]. Existe bastante evidencia de los efectos moduladores de los parásitos, aunque la mayoría de ellos, causan invasión y daño en algunos órganos del hospedero.

Taenia crassiceps

Es un parásito que se encuentra como adulto en el intestino de los cánidos y en estado larvario (metacéstodo) en el músculo, cavidad pleural y peritoneo de roedores. Una característica interesante de este parásito es que, en su etapa larvaria, puede reproducirse asexualmente, la cual permite que pueda permanecer en su hospedero por largos periodos.

En la infección experimental con *T. crassiceps*, la inoculación de 10-20 metacestodos en la cavidad peritoneal de los ratones, lleva al cabo de 6-8 semanas a la aparición de cientos de parásitos. La etapa larvaria de *T. crassiceps* es inocua en humanos, su tamaño es macroscópico y

no causa la muerte de su hospedero. La respuesta inmune de la infección por *T. crassiceps* se divide en dos etapas: aguda y crónica. Al inicio de la infección se presenta una respuesta Th1 con incremento de IFN-γ, ON e IgG2a, que posterior a la semana 3 se reemplaza por una respuesta Th2 con incremento de IL-4 e IL-13, como también de IgG1 e IgE. Posterior a la semana 5, se incrementa la carga parasitaria de forma considerable y la población de AAMϕS [32, 33]. Para comprobar el efecto modulador de *T. crassiceps* en un ambiente inflamatorio, en un trabajo anterior, quisimos probar sí la infección previa con *T. crassiceps* podía regular la T1D. Para lo cual, utilizamos dos cepas de ratones, una cepa susceptible a la infección y una resistente, BALB/c y C57BL/6Hsd, las cuales fueron infectados con 20 metacestodos de *T. crassiceps* via intraperitoneal (i.p). Después de 6 semanas de infección, los animales fueron inducidos con MLD-STZ. Evaluamos la glucemia semanalmente por 6 semanas posteriores a la inducción de la diabetes. También evaluamos la incidencia de la diabetes, el infiltrado celular de los islotes pancreáticos (insulitis), citocinas como IL-4 y TNF-α; además de la presencia de AAMϕS. Lo que encontramos es que efectivamente los ratones BALB/c son más susceptibles a la infección que los ratones C57BL/6, presentándose mayores cargas parasitarias; pero, los ratones C57BL/6 son más susceptibles a la T1DM que los BALB/c, con mayor infiltrado celular en páncreas. Encontramos que en ambas cepas, la presencia de *T. crassiceps* disminuye de forma considerable, la incidencia, el infiltrado celular, se incrementan la IL-4 en suero, no hay presencia de Treg y los AAMϕS aparecen de forma significativa, en comparación con los diabéticos, comprobando que la infección con *T. crassiceps* puede revertir el efecto inflamatorio de la diabetes y, sugiriendo que los AAMϕS pueden jugar un papel importante en la protección frente a la T1DM [24]. En un estudio reciente, la transferencia de AAMϕS activados por IL-4 e IL-13 en ratones diabéticos presentaron menor daño en riñones, redujeron la hiperglicemia y la insulitis del páncreas, mostrando que los AAMϕS protegen contra la diabetes [34].

HIPOTESIS

El tratamiento con el antígeno derivado de *T. crassiceps* (TcS o TcES) puede reducir la hiperglucemia y el daño en los islotes de Langerhans, por medio del incremento de la respuesta Th2 y de AAMϕS. Posiblemente otras células como las MDSCs colaboren en esta regulación.

OBJETIVO

PRINCIPAL

- **Determinar si la administración de los antígenos de *Taenia crassiceps* alteran el desarrollo de la T1DM experimental y si está relacionado a la presencia de AAMϕS.**

Particulares

1. Comprobar si el tratamiento con el TcS reduce la hiperglucemia e incidencia de la T1DM y si existen diferencias con respecto a la infección.
2. Comprobar si la administración del TcES regula la T1DM y si hay cambios con respecto al tratamiento anterior.
3. Conocer si la modulación de la T1DM esperada está asociada a la generación de los AAMϕS y MDSCs.

MATERIALES Y METODOS

Ratones

Se utilizaron ratones machos de la cepa BALB/cAnN de 6 a 8 semanas de edad. Los animales se encuentran en condiciones libres de patógenos en el bioterio de la FES Iztacala, de acuerdo con las normas institucionales y nacionales.

Infección de los Ratones

Los metacestodos de *T. crassiceps* (de la cepa ORF) se obtuvieron de ratones previamente infectados de 4-6 semanas de infección, los parásitos se lavaron con solución salina estéril (Pisa) dos veces, y los metacestodos fueron seleccionados (~ de 2.0 mm de diámetro y sin gemación). Se inyectaron 20 metacestodos via i.p. a ratones machos de 6- 8 semanas de edad.

Antígeno Soluble de *T. crassiceps* (TcS)

El antígeno soluble se generó de metacestodos de *T. crassiceps* provenientes de la cavidad peritoneal de ratones BALB/c de 6-8 semanas de infección, obtenidos en esterilidad y lavados exhaustivamente con solución salina. Posteriormente, se hizo un homogenizado utilizando un sonicador en esterilidad, se agregó un inhibidor de proteasas (25 µl/10 ml) y se centrifugaron a 10000 rpm por 30 min a 4°C, para obtener el sobrenadante. Se cuantificaron las proteínas por medio de un ensayo de Bradford (Biorad) y se mantuvo en congelación a -80°C.

Antígeno de *T. crassiceps* Excretado/Secretado (TcES)

Se preparó con metacéstodos obtenidos de la cavidad peritoneal en condiciones estériles de ratones infectados BALB/c de 6-8 semanas de infección. Posteriormente, se lavaron 3 veces consecutivas con solución de PBS estéril. Los metacéstodos fueron depositados en una placa de

6 pozos (Costar, Cambridge, MA) por 24 horas a 37°C y 5% de CO_2. El sobrenadante fue colectado y centrifugado a 1000g x 10 minutos. Consecutivamente, el sobrenadante se colocó en un tubo Amicon Ultra de 50 KDa (Millipore) para recolectar las proteínas superiores a 50 KDa, se centrifugaron a 2000g por 30 minutos. Se colocaron inhibidores de proteasa a la fracción ≥50KDa y las muestras fueron almacenadas a -80°C hasta su uso. La concentración de proteína fue determinada por el ensayo de Bradford (Biorad).

Tratamientos Experimentales

Se realizaron varios tratamientos experimentales que se describen más detalladamente en la siguiente tabla:

Tratamiento	Descripción	Grupos experimentales
STZ	Los ratones fueron inyectados con MLD-STZ para la inducción de T1DM. Durante 5 dias consecutivos se les inyecto a los animales dosis de STZ via i.p. (Sigma-Aldrich;45 mg/kg) diluida en 0.1M de citrato de sodio a pH de 4.5. [8].	STZ
STZ/TcS	Ratones con MLD-STZ y tratados con antígeno soluble de *T. crassiceps* (TcS) se subdividieron en:	
	Ratones que recibieron inyecciones i.p de TcS (50 µg), 3 veces por semana, únicamente una semana antes de la inducción de la T1DM y durante la semana de la inducción de la diabetes.	STZ/TcS-1
	Ratones tratados con inyecciones i.p. del TcS (50 µg), 3 veces por semana, de manera constante, una semana antes de MLD-STZ y durante las 6 semana posteriores.	STZ/TcS-2
	Aquellos que fueron tratados con inyecciones i.p de TcS (50 µg), 3 veces por semana, comenzando una semana después de la inducción de la T1D y durante las 6 semanas posteriores.	STZ/TcS-3
	Animales con inyecciones i.p. de TcS (100 µg), 3 veces por semana, con el mismo esquema que los anteriores.	STZ/TcS-4
	Finalmente, aquellos de recibieron i.p. inyecciones de TcS (200 µg), 3 veces por semana, iniciando una semana después de la inducción de la T1D y durante las 6 semanas posteriores.	STZ/TcS-5
STZ/TcES	Animales tratados con inyecciones i.p de TcES e inducidos con MLD-STZ:	
	Ratones tratados con inyecciones i.p. del TcES (50 µg), 3 veces por semana, de manera constante, una semana antes de MLD-STZ y durante las 6 semana posteriores.	STZ/TcES-1

	Aquellos que fueron tratados con inyecciones i.p de TcES (50 µg), 3 veces por semana, comenzando una semana después de la inducción de la T1D y durante las 6 semanas posteriores.	STZ/TcES-2
	Animales con inyecciones i.p. de TcES (100 µg), 3 veces por semana, con el mismo esquema que los anteriores	STZ/TcES-3
	Finalmente, aquellos de recibieron i.p. inyecciones de TcES (200 µg), 3 veces por semana, iniciando una semana después de la inducción de la T1D y durante las 6 semanas posteriores.	STZ/TcES-4
Liposomas	Para conocer el papel de los AAMos en la T1DM, se realizó el siguiente experimento. Ratones de 6-8 semanas de edad fueron infectados o no, con 20 metacestodos de *T. crassiceps* y después de 6 semanas, se inyectaron con MLD-STZ para inducir T1DM.	
	Los animales sin infección se trataron con liposomas con PBS o clodronato (Cl), desde la segunda semana posterior a la inducción de la T1DM (3 veces por semana, 200 µl/ratón) hasta la sexta semana.	STZ Cl STZ PBS
	Los animales infectados, fueron inducidos con MLD-STZ. En la segunda semana después de la T1DM, los ratones fueron tratados con inyecciones i.p. de liposomas con Cl o PBS (200 µl/ratón, 3 veces por semana) durante 4 semanas posteriores a la inducción de la T1D.	STZ/Tc Cl STZ/Tc PBS
	Un tratamiento similar se realizó con animales tratados con antígeno soluble (siguiendo el mismo esquema que los animales STZ/TcS-2).	STZ/TcS Cl STZ/TcS PBS
Untreated	Animales que no recibieron tratamiento de ningún tipo.	Untreated

Monitoreo de glucosa

Los ratones fueron monitoreados semanalmente para ver el desarrollo de la diabetes. Se midió la glucosa en sangre con ayuda de un glucómetro (Accu-Chek), posterior a 4-6 horas de ayuno. la glucemia superior a 200mg/dl en dos mediciones consecutivas, son considerados diabéticos. Se registró la incidencia de diabetes en todos los grupos de ratones.

Prueba de tolerancia a la glucosa

A la sexta semana posterior a la inducción de la diabetes, los animales se dejaron en ayuno por 4-6 horas. Posteriormente se inyecto glucosa-D (1.5 mg/kg) vía i.p. y se obtuvo sangre de la cola de los ratones por periodos de 30, 60 y 120 minutos por medio de un glucómetro (Accucheck).

Obtención de células peritoneales (PECS)

En la sexta semana posterior a la inducción de la diabetes los ratones fueron sacrificados. Se obtuvieron las células peritoneales (PECs) inyectando en el peritoneo 10 ml de solución salina i.p. dando un ligero masaje y extrayéndolas con ayuda de una jeringa. Se colocaron en tubos cónicos estériles de 15 ml. Estas células fueron lavadas tres veces, posteriormente, fueron resuspendidas en solución de Boyle (0.17M Tris y 0.16M de cloruro de amonio) para lisar los eritrocitos. Se centrifugaron a 3000 rpm durante 10 minutos. El sobrenadante se decantó y el botón se resuspendió. Después de dos lavados, las células viables fueron contadas por exclusión de azul de tripano en una cámara de Neubauer. Se ajustó la concentración celular a 1 x 10^6 células/ml.

Citometría de Flujo

Se bloquearon los receptores Fc con anti-mouse CD16/CD32 (Biolegend, CA,USA) en las PECS, se centrifugaron a 2500rpm durante 10 minutos, se decantaron y se obtuvo la pastilla. Se emplearon anticuerpos monoclonales F4/80-FITC, MMR-FITC, F4/80-APC, PDL-1-PE, PDL-2-PE, CD11b-APC, CD11c-FITC, IL-4Rα-PE y Gr1-PE se diluyeron a una concentración de 0.2µg/1x10^6 células y se incubaron por 30 minutos a 4°C en oscuridad. Se lavaron con 1ml de buffer de fosfatos y se centrifugaron a 2500 rpm durante 10 minutos para eliminar los anticuerpos no adheridos. Se fijaron con 500µl de paraformaldehido. Las células fueron analizadas en un citometro FACSCalibur utilizando el software Cell Quest (Becton Dickinson).

ELISA

Todos los ratones se sangraron una vez por semana por medio de una incisión caudal, la sangre se colectó en tubos eppendorf de 1.8 ml. Posteriormente se centrifugo a 3000rpm durante 10 minutos (dos veces), obteniendo suero, el cual se mantuvo en congelación hasta su uso. Los niveles de citocinas: IFN-γ e IL-4 se evaluaron por medio de la técnica de ELISA-sandwich de acuerdo a las instrucciones del fabricante (Pharmigen, Preprotech).

El anticuerpo de captura se diluyo a una concentración de 2µg/ml en solución de pegado (Na_2HPO_4 0.1Ma pH 9.0, Baker) adicionando 50µl/pozo a placas de 96 pozos para ELISA (Nunc Maxisorp), se cubrieron y se dejaron reposar toda la noche a 4°C.

Al día siguiente, las placas se lavaron 3 veces con solución de lavado (PBS-tween-20 al 0.05%, Sigma) y se les agrego 200µl de buffer de bloqueo (PBS 1X con suero fetal bovino al 10%) dejando incubar a 37°C durante 2 horas. Al finalizar el tiempo de incubación, las placas se enjuagaron con solución de lavado dos veces y se colocaron 20µl de muestra por duplicado. Para la curva se utilizó la citocina recombinante de nuestro interés y se realizaron diluciones a la mitad en buffer de bloqueo. Las placas se incubaron a 4°C durante toda la noche.

Al tercer día, las placas se dejaron reposar a temperatura ambiente por 10 minutos y lavaron en solución de lavado 4 veces, se les agregó el anticuerpo de detección marcado con biotina correspondiente para cada citocina a una concentración de 2µg/ml en buffer de bloqueo y se incubaron a 37°C por 1hr. Por 5 veces se lavaron en solución, y se adicionó 5µl/ pozo del conjugado de estreptoavidina peroxidada (Biorad) a una dilución de 1: 2000 en buffer de bloqueo y se incubarón a 37°C por 45 minutos. Se lavaron 6 veces y se agregó 100 µl/pozo de sustrato ABTS (0.5% ABTS en 0.1M ácido cítrico a pH de 4.5con NaOH más 1% de H_2O_2 al 3%, Sigma). La densidad óptica se determinó entre los primeros 30 minutos en un lector de ELISA (SpectraMax 250, Molecular Devices) a 405 nm.

Histología

Los páncreas de los ratones de cada grupo fueron removidos y fijados en paraformaldehido al 4% y se mantuvieron a 4°C por mínimo 2 horas; posteriormente para el procesamiento en parafina los páncreas se lavaron con agua corriente durante 30 minutos, seguido de alcoholes graduales (comenzando por el OH-70%, OH-80%, OH-90% y OH-100% por 30 min en cada uno) y por último en butanol por 2 hrs. Se fijaron en parafina (Kendall) y se cortaron en secciones de 5µM de grosor (el tejido se extendió con Ruyter).

Las laminillas se dejaron reposar por mínimo 30 min a 60° para mejorar el pegado, seguido de un tren de tinción: Xilol 10´, OH-Xilol 3´, OH 100% 3´, OH 90% 2´, OH-80% 2´, OH-70% 1´, H_20 3´, Hematoxilina de Harris 2-5´, H_20 5´, OH-Acido lavado, H_20 3´, Eosina alcoholica 1-3´, H_20 5´, OH-70% 1´, OH-80% 1´, OH-90% 1´, OH-100% 5´, y Xilol 10´. El montaje de las laminillas fue con Entellan (Baker), para posteriormente observarlas en el microscopio óptico (Zeiss).

Los islotes por ratón se observaron, y el grado de infiltración celular fue registrado independientemente, usando la siguiente escala: 0 sin infiltrado, normal; peri-insulitis (células mononucleares alrededor de los islotes y ductos, pero sin infiltrado); moderada insulitis (infiltración celular <50% del islote); 3, severa insulitis (>50% de infiltración celular de los islotes y perdida de arquitectura de los mismos). Se tomaron fotos con ayuda de una cámara (Zeiss) y se visualizaron por el programa Axio Vision Rel 4.6 (Zeiss). La evaluación de la insulitis fue representativa de 10 ratones por grupo (al menos 100 islotes).

Análisis estadístico

Los datos fueron analizados utilizando GraphPadPrism Software (versión 5.0; GraphPad Software, San Diego, CA, USA). Para la comparación entre los grupos, se realizó la prueba Mann-Whitney U para las diferencias con los datos no-parámetricos. Experimentos con múltiples grupos se realizó la prueba Kruskal-Wallis seguido de la prueba de comparación de Dunn. Los datos se presentan como media ± SEM; Valores de $P<0.005$ fueron tomados como estadísticamente significativos ($P\leq0.05$*; $P\leq0.01$**; $P\leq0.001$***).

RESULTADOS

Los resultados se consultan en el siguiente artículo.

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Research Article

Taenia crassiceps Antigens Control Experimental Type 1 Diabetes by Inducing Alternatively Activated Macrophages

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Type 1 diabetes (T1D) is an autoimmune disease caused by the selective destruction of the pancreatic β-cells, causing inability to produce insulin. Proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ, IL-12, IL-17, and NO can be released by CD4 and CD8$^+$ lymphocytes as well as by classically activated macrophages (CAMϕs), which are important in the development of T1D. Helminth infections have been shown to prevent T1D, mainly through Th2-biased responses and increased recruitment of regulatory cell populations. Previously, we have shown that *Taenia crassiceps* infection in mice significantly reduces hyperglycemia, insulitis, and the incidence of T1D. In this study, we determined whether *T. crassiceps*-derived products such as soluble (TcS) or excreted/secreted (TcES) antigens might have a beneficial influence on the development of experimental T1D. Treatment with different doses before or after induction of T1D was analyzed. Mice that were pretreated with TcS were unable to develop T1D, whereas those receiving TcES early after T1D induction displayed significantly reduced insulitis and hyperglycemia along with increased recruitment of alternatively activated macrophages (AAMϕs) and myeloid-derived suppressor cells (MDSCs). Finally, we examined the modulatory role of AAMϕs on T1D by depleting macrophages with clodronate-loaded liposomes, demonstrating that AAMϕs are key cells in T1D regulation.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by the selective destruction of the pancreatic β-cells, lowering insulin production [1]. The absence of insulin disturbs the regulation of blood glucose concentration, resulting in severe hyperglycemia. The onset of diabetes involves both genetic and environmental factors [2]. Proinflammatory cytokines such as IL-1β, IL-6, TNF-α, IFN-γ, IL-12, IL-17, and nitric oxide (NO) can be released by CD4$^+$ and CD8$^+$ T lymphocytes as well as by CAMϕs, which seems to be important in the development of insulitis and the death of beta cells [3, 4]. Experimental rodent models such as nonobese diabetic mice (NOD) or multiple low doses of streptozotocin (MLD-STZ) have provided new knowledge and strategies to regulate this disease [5].

During the last few years, anti-inflammatory response has been proposed as a helpful way to regulate the immune system during autoimmune diseases. For example, in NOD mice, the administration of IL-4 or IL-10 may prevent the development of diabetes [6]. In addition, helminth infections have been shown to prevent T1D development. The mechanisms of this effect may consist of inducing a strong Th2-type response and increasing regulatory cell populations, such as Treg, MDSCs, and AAMϕs, that exert regulatory effects on the immune system of their host [7]. Infections with *Schistosoma mansoni* [8], *Heligmosomoides polygyrus* [9, 10], *Litomosoides sigmodontis* [11], *Trichinella spiralis* [9], *Nippostrongylus brasiliensis*, and *Strongyloides venezuelensis* [12] can provide different levels of protection against the onset of T1D in NOD and MLD-STZ-induced T1D mice, but all these parasites may induce dangerous side effects in their hosts. For example, *Schistosoma mansoni*, *Litomosoides sigmodontis*, and *Trichinella spiralis* have complex life cycles that include migration through several tissues including lungs, bladders, and muscle where these

parasites cause damage. Previously, we have shown that infection with the cestode *Taenia crassiceps* significantly decreases hyperglycemia, insulitis and, consequently, the incidence of T1D in mice treated with MLD-STZ. Moreover, higher levels of IL-4 in sera and an increase in the AAMϕ population were induced as well, suggesting that this population could be important in protection against T1D [13].

AAMϕs are induced by Th2 responses, such as those induced by helminth infections [14]. AAMϕs can produce IL-10 and TGF-β and downregulate levels of proinflammatory cytokines. Additionally, AAMϕs express suppressive molecules such as PDL-1 and PDL-2 (PD-1 ligands), which are both associated with inhibiting proliferative responses [15–17]. Likewise, AAMϕs express arginase-1, which induces a shift in arginine metabolism towards the production of L-ornithine, a precursor for polyamines and collagen, important molecules in wound healing [18, 19]. In contrast, CAMϕs produce iNOS, which converts L-arginine into ROS and NO. Free radicals and NO are critical in inducing damage to β-cells [15, 19]. Interestingly, in many studies, infection with helminths or treatment with their antigens led to an increase in the numbers of AAMϕs. For example, *S. mansoni* infection or treatment with soluble worm antigen (SWA) or soluble egg antigen (SEA) brings about a reduction in the inflammatory response in NOD mice, which has been correlated with increasing AAMϕ and Treg populations [20–22]. In another study, E/S products from *Fasciola hepatica* (FhES) reduced pancreatic islet damage and hyperglycemia in NOD mice, increasing AAMϕs in the pancreas and pancreatic lymph nodes [23]. Furthermore, adoptive transfer of AAMϕs induced in vitro by IL-4 and IL-13 in NOD mice decreased hyperglycemia and insulitis, suggesting a protective effect in this population [24]. Similarly, another study using the MLD-STZ model revealed that angiogenesis and the wound healing process were more effective if AAMϕs were present [25]. However, in most of these studies, helminths or their products were administered before T1D onset, leaving open the question of whether such treatments may be useful as a therapeutic alternative.

Another important population also able to suppress immune responses is the myeloid-derived suppressor cell (MDSC) population. MDSCs are a heterogeneous population of myeloid progenitor cells and immature myeloid cells that delay their maturation and can be differentiated by the expression of different markers such as CD11b and Gr1 [26]. Two subsets of MDSCs were recently defined: monocytic MDSCs, which express $CD11b^{+}Ly6C^{high}Ly6G^{-}$, and granulocytic MDSCs, which express $CD11b^{+}Ly6G^{+}Ly6C^{low}$; each population has a different function in cancer, parasitic infections, and autoimmunity [26, 27]. However, their role in T1D development is largely unknown.

The purpose of this study was to determine whether soluble or excreted/secreted products of *T. crassiceps* could protect against T1D induced by MLD-STZ. Additionally, we evaluated the protective role of AAMϕs and MDSCs induced by *T. crassiceps* and their products in T1D development.

2. Materials and Methods

2.1. Mice. Six- to eight-week-old male BALB/cAnN mice were purchased from Harlan Laboratories (Mexico) and were maintained in a pathogen-free environment at the animal facility at FES-Iztacala, UNAM, in accordance with institutional and national guidelines.

2.2. Parasites and Antigen Preparation/Quantification. Metacestodes of *Taenia crassiceps* were harvested in sterile conditions from the peritoneal cavity of male BALB/cAnN mice after 2–4 months of infection and were washed 4 times with ice-cold, sterile PBS.

(i) *T. crassiceps* soluble antigen (TcS) was prepared by homogenizing whole metacestodes (10 ml volume) in 2 rounds of 3 seconds each by using a homogenizer (Polytron, Kinematica). The homogenates were centrifuged at 2000 ×g for 20 minutes at 4°C, and the supernatants, which contained PBS-soluble antigens, were collected and frozen at −80°C until further use. Protein concentration was determined using a Bradford protein assay kit (BioRad). Preparation of TcS was similar to that described in [28].

(ii) *T crassiceps* excreted/secreted products (TcES) were prepared as described elsewhere by Terrazas et al. [29]; briefly, metacestodes were harvested in sterile conditions following 3 washes with sterile PBS. Metacestodes were seeded in 6-well plates (Costar, Cambridge, MA) for 24 hours at 37°C and 5% CO_2. Supernatants were collected and centrifuged at 1000 ×g for 10 minutes. Subsequently, the upper fraction was concentrated using 50 kDa Amicon Ultra Filter tubes (Millipore) and further centrifuged at 2000 ×g for 30 minutes. Protease inhibitors were added to the ≥50 kDa fraction, and samples were stored at −80°C until further use. Protein concentration was determined using a Bradford protein assay kit (BioRad).

2.3. Treatments. Different experimental groups as detailed in Table 1 were used.

2.4. Blood Glucose Monitoring. Blood glucose was measured at 0, 1, 3, and 6 weeks after T1D induction using an Accu-Check Advantage glucometer (Roche Diagnostics) in animals that had been fasted for 6 hours. Animals were considered diabetic when fasting blood glucose was greater than 200 mg/dl.

2.4.1. Glucose Tolerance Test. At 6 weeks after induction of T1D, mice were subjected to an intraperitoneal glucose tolerance test in order to establish the effects of their diabetes on glucose metabolism. The mice were fasted for 6 hours prior to sample collection. A basal blood sample (time 0) was collected by tail snip, and plasma glucose was evaluated using an Accu-Check Advantage glucometer. Mice were injected i.p. with filtered D-glucose (1.5 mg/kg). Glucose levels were evaluated again at the 30-, 60-, and 120-minute time points.

Table 1: Description of experimental groups.

Treatment	Description	Groups
STZ	Diabetic mice were induced with multiple low doses of streptozotocin (MLD-STZ). They received during five days consecutive intraperitoneal injections (i.p) of STZ (Sigma-Aldrich; 45 mg/kg) dissolved in 0.1 M sodium citrate, pH 4.5 [30]	STZ
STZ/TcS	Mice with MLD-STZ and injections of *T. crassiceps* soluble antigens	
	With 50 μg of TcS i.p. 3 times for a week, one week before, and during the week of induction with MLD-STZ	STZ/TcS-1
	Treated constantly with 50 μg i.p. 3 times per week, one week before, during treatment with MLD-STZ, and for 6 weeks post induction until euthanasia	STZ/TcS-2
	Treated with 50 μg i.p. 3 times per week, starting 1 week post induction of T1D and continuing until sacrifice	STZ/TcS-3
	Treated with 100 μg i.p. 3 times per week during 1 week post induction of T1D and for 6 weeks afterward	STZ/TcS-4
	Treated with 200 μg i.p. 3 times per week, starting 1 week post induction T1D and continuing for 6 weeks	STZ/TcS-5
STZ/TcES	Animals treated with injections of *T. crassiceps* excreted/secreted antigens with MLD-STZ	
	Treated with 50 μg i.p. 3 times per week, one week before, during treatment with MLD-STZ, and for 6 weeks post induction	STZ/TcES-1
	Treated with 50 μg i.p. 3 times per week started 1 week post induction of T1D for the rest of the treatment period	STZ/TcES-2
	Treated with 100 μg i.p. 3 times per week since first week post induction of T1D for the rest of the treatment period	STZ/TcES-3
	Treated with 200 μg i.p. 3 times per week, started one week after induction of T1D and during 6 weeks	STZ/TcES-4
Liposomes	Six- to eight-week-old mice were infected i.p. with 20 cysticerci, and then we waited 6 weeks post infection to induce diabetes by MLD-STZ. We chose this time period to induce diabetes because we know by a previous report from our group that the change in the immune response to a Th2 response and the appearance of AAMϕs have been established to occur in the sixth to eighth week post infection [31]. Macrophages were depleted in vivo using dichloromethylene diphosphonate (clodronate) encapsulated in liposomes. Treatment of liposome injections was followed as Reyes et al. [31]	
	In the second week after T1D-induction, mice were treated with i.p. injections of clodronate liposomes (Cl) or PBS liposomes (200 μl/mouse i.p. 3 times/week) for 5 weeks post T1D induction	STZ Cl STZ PBS
	Two weeks after T1D induction by MLD-STZ, *T. crassiceps*-infected and *T. crassiceps*-uninfected mice were injected i.p. with Cl liposomes and PBS liposomes (200 μl/mouse i.p. 3 times/week) for 5 weeks post T1D induction	STZ/Tc Cl STZ/Tc PBS
	An equivalent treatment with clodronate liposomes was done in TcSA-treated Balb/c mice. Animals were injected on a similar schedule to STZ/TcS-2, the second week before T1D induction and continuing for 4 weeks post induction. Blood glucose levels were measured for 4 weeks	STZ/TcS Cl STZ/TcS PBS
Untreated	Receiving neither antigens nor MLD-STZ	Untreated

2.4.2. Histology. Pancreases from all groups were collected 6 weeks after the induction of diabetes. The tissues were processed and embedded in paraffin, and 5 μm sections were cut for analysis. Thin sections were stained with hematoxylin and eosin (H&E) and evaluated microscopically for the presence of insulitis using the following scoring system: noninfiltrated (healthy islets), peri-insulitis (lymphocytes at the periphery of the islets), insulitis 20% (insulitis into the interior of the islets $\leq$20%), and insulitis 40% (insulitis into the interior of the islets $\geq$40% with damage to islet architecture). The insulitis evaluation shown is representative of 10 mice per group (at least 100 islets).

2.4.3. Cytokine ELISAs. Peripheral blood was collected from tail snips at 1, 3, and 6 weeks post induction. Serum IL-4 and TNF-α were measured by sandwich ELISA using a commercial kit purchased from Peprotech, Mexico.

2.4.4. Flow Cytometry. Peritoneal exudate cells (PECs) were obtained from the peritoneal cavity from distinct groups of mice 6 weeks p.i. of MLD-STZ. The cells were washed twice with physiological saline solution, and the red blood cells were lysed by resuspending the cells in Boyle's solution (0.17 M Tris and 0.16 M ammonium chloride). Following two washes, the viable cells were counted by trypan blue exclusion with a Neubauer hemocytometer. The PECs were adjusted (1×10^6 cells), and Fc receptors were blocked with anti-mouse CD16/CD32 (Biolegend, CA, USA) and then stained with APC-F4/80, APC-CD11b, FITC-CD11c, FITC-MMR, PE-PDL-1, PE-PDL-2, PE-IL-4Rα, and PE-Gr1 (all from Biolegend, CA, USA) and incubated for 30 minutes at 4°C in FACSFlow staining buffer (Becton Dickinson). The cells were analyzed using a FACsCalibur and CellQuest Software (Becton Dickinson).

2.4.5. Statistical Analysis. Data were tested for statistical significance using GraphPad Prism software (version 5.0; Graphpad Software, San Diego, CA, USA). For comparisons between the two groups, the Mann–Whitney *U* test was applied to test differences with nonparametric data. Experiments with multiple groups were tested by the Kruskal-Wallis test followed by Dunn's multiple comparison

test. Data are presented as mean ± SEM; p value < 0.05 was considered statistically significant ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$).

3. Results

3.1. T. crassiceps Soluble Antigen Reduces Experimental T1D in Mice Only If the Treatment Remains Constant. Our first goal was to test whether soluble *T. crassiceps* antigens may protect against T1D through prior or constant exposure. Thus, mice were treated i.p. with 50 μg of TcS 3 times a week, starting one week before T1D induction and continuing for six consecutive weeks post T1D induction until euthanasia (STZ/TcS-2), or with a dose of 50 μg of soluble antigen one week before induction of MLD-STZ, 3 times in a week (STZ/TcS-1) (Figure 1(a)). Blood glucose was measured once a week. Figure 1(b) shows that STZ/TcS-2 mice had lower blood glucose levels than STZ at 3 and 6 weeks after disease induction; however, STZ/TcS-1 did not present this effect. Surprisingly, STZ/TcS-2 mice, which had a constant exposure to TcS antigen, downregulated the hyperglycemia at borderline of nonpathological levels (200 mg/dl). Moreover, while the percentage of mice free of diabetes in the STZ group was 0% (with diabetes defined as glucose levels > 200 mg/dl), the STZ/TcS-2 group displayed a significant reduction in T1D (50%), whereas STZ/TcS-1 had a positive effect in only 25% of mice (Figure 1(c)). Additionally, we evaluated glucose tolerance in these mice by injecting them i.p. with D-glucose (1.5 mg/kg), and their blood glucose was evaluated at different times. Hyperglycemia was significantly reduced in mice receiving TcS constantly compared with the STZ and STZ/TcS-1 groups, which showed hyperglycemia up to 300 mg/dl (Figure 1(d)).

3.2. Constant Exposure to TcS Reduces Insulitis in T1D-Induced Mice. At week 6 post T1D induction, mice were euthanized, and their pancreases were removed and processed for histology to evaluate insulitis. The STZ and STZ/TcS-1 groups revealed extensive and severe peri-insulitis and insulitis at the islets of Langerhans, with clear loss of islet architecture, while mice constantly exposed to TcS antigen (STZ/TcS-2) displayed a significant reduction in the number of infiltrated islets and the structure remained unmodified in most of them (Figures 2(a) and 2(b)).

3.3. Constant TcS Treatment in Diabetic Mice Induces High Levels of IL-4. To evaluate whether TcS treatment might be able to modify inflammatory response, circulating cytokine levels in sera of the different groups were measured. TNF-α, an important cytokine related to pancreatic islet damage, displayed similar levels between groups, but at the sixth week after T1D induction, we observed lightly increasing levels of TNF-α in STZ mice (Figure 3(a)). In contrast, IL-4 was significantly elevated in STZ/TcS-2 mice since the first week of treatment, whereas STZ and STZ/TcS-1 mice displayed lower levels of this cytokine (Figure 3(b)).

3.4. Constant TcS Exposure Recruits AAMϕs. Next, we examined whether TcS exposure might favor the recruitment of AAMϕs. PECs were analyzed by flow cytometry, and we evaluated $FSC^{high}SSC^{high}F4/80^{+}$ cells to look for AAMϕ markers. As shown in Figure 4, STZ-treated and STZ-untreated mice had reduced percentages of AAMϕs, while STZ/TcS-2 displayed elevated percentages of AAMϕs, given that the expression levels of MMR, IL-4Rα, PDL-1, and PDL-2 were significantly upregulated in STZ/TcS-2-treated mice compared to STZ-treated mice.

3.5. Treatment with TcES Reduces the Incidence of T1D. A major question was whether exposure to *T. crassiceps* products early after T1D induction could be effective to protect diabetic mice. We used 3 different variables with the same treatment regime as described before: mice were injected i.p. with 50 μg (STZ/TcS-3), 100 μg (STZ/TcS-4), or 200 μg (STZ/TcS-5) of TcS started in the first week post T1D induction (Figure 5(a)). In addition, we tested TcES treatment in a constant form, 50 μg/dose for 1 week before and 6 weeks after induction of T1D (STZ/TcES-1) or as a post induction treatment 50, 100, or 200 μg beginning after the first week post T1D induction (STZ/TcES-2, STZ/TcES-3, and STZ/TcES-4, resp.) using the scheme described before (Figure 5(a)). We found that TcS treatment did not show a protective effect at any dose when the animals were exposed to *T. crassiceps*-derived antigens one week post induction of diabetes (Figure 5(b)). Also, constant TcES (STZ/TcES-1) treatment was insufficient to reduce hyperglycemia. In contrast, a 200 μg/mouse dose of TcES antigen (STZ/TcES-4) was more effective to reduce the high levels of glucose in diabetic mice in a post induction scheme (Figure 5(c)). Additionally, the percentage of mice free of diabetes in the STZ group at the 6th week was zero, whereas in STZ/TcES-4, the percentage was 50%, as in the STZ/TcS-2 (Figure 5(d)).

Next, we evaluated both insulitis in the pancreas and the damage score in the islets. Figures 5(e) and 5(f) show that the STZ/TcES-4 group had significantly less damage in the pancreas and therefore a lower damage score; most of the islets showed neither infiltration nor peri-insular infiltration, as opposed to the STZ group, which displayed severe damage in the islets and developed insulitis in 20–40%.

STZ/TcES-4 treatment also significantly increased the expression of AAMϕ markers such as PDL-2 and MMR compared to the levels in STZ-induced mice (Figure 6(a)).

3.6. Exposure to T. crassiceps-Derived Products after T1D Induction Increases the Population of $FSC^{high}Gr1^{+}CD11b^{+}$ Cells. To examine whether exposure to *T. crassiceps* antigens that were effective in decreasing hyperglycemia (STZ/TcS-2 and STZ/TcES-4) in diabetic mice could modify the recruitment of MDSCs, we evaluated the expression of CD11b and Gr1 markers in peritoneal cells by flow cytometry. As shown in Figure 6(b), STZ/TcS-2 and STZ/TcES-4 treatment led to the recruitment of significantly higher percentages of $FSC^{high}SSC^{high}CD11b^{+}Gr1^{+}$ cells (34% ± 7 and 75% ± 3, resp.), compared with STZ mice (3% ± 0.9) and untreated mice (1.7 ± 0.07%). These results suggest a possible positive role for the $CD11b^{+}Gr1^{+}$ population as well as AAMϕs in regulating T1D development.

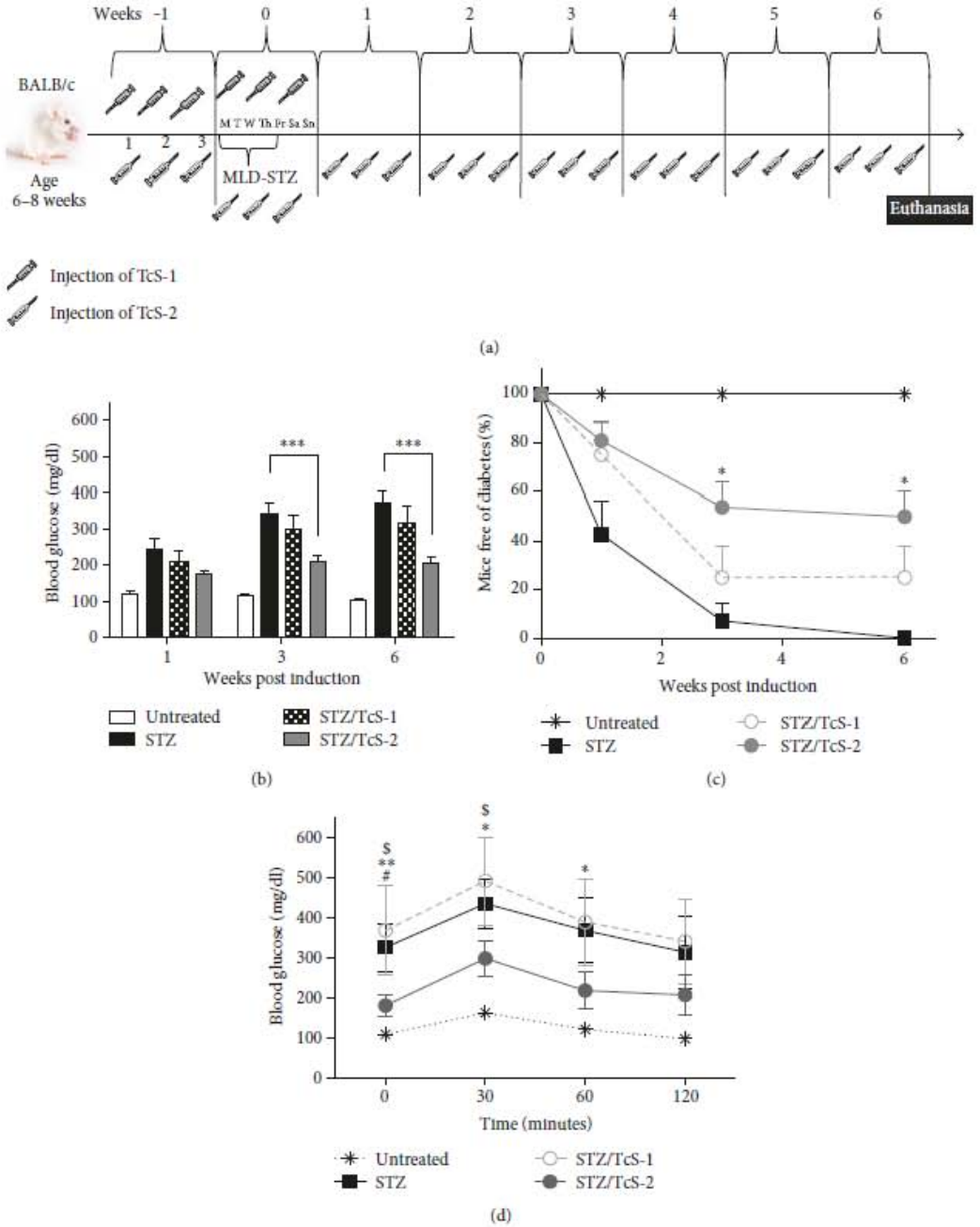


FIGURE 1: *T. crassiceps* soluble antigens were protective against T1D development only when antigen was constantly injected (STZ/TcS-2). (a) Methodology diagram illustrating treatment with *T. crassiceps* antigen. (b) Blood glucose levels for all groups. STZ/TcS-2 treatment was able to significantly reduce glycemia to normal levels (≤200 mg/dl) compared with STZ group. (c) Percentage of mice free of diabetes; mice with glycemia higher than 200 mg/dl were considered diabetic mice, whereas those with levels below 200 mg/dl were considered free of diabetes. The STZ/TcS-2-treated group showed a lower percentage of T1D incidence than the STZ and STZ/TcS-1 groups. (d) Glucose tolerance test for all groups. *Differences between STZ versus untreated group. #Differences between STZ/TcS-1 versus untreated group. $Differences between STZ/TcS-2 versus Untreated group. The data represent at least 3 independent experiments; $N = 5$ mice per group. For comparisons between two groups, the Mann–Whitney U test was applied to test differences with nonparametric data. Experiments with multiple groups were tested by the Kruskal-Wallis test followed by Dunn's multiple comparison test. Mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

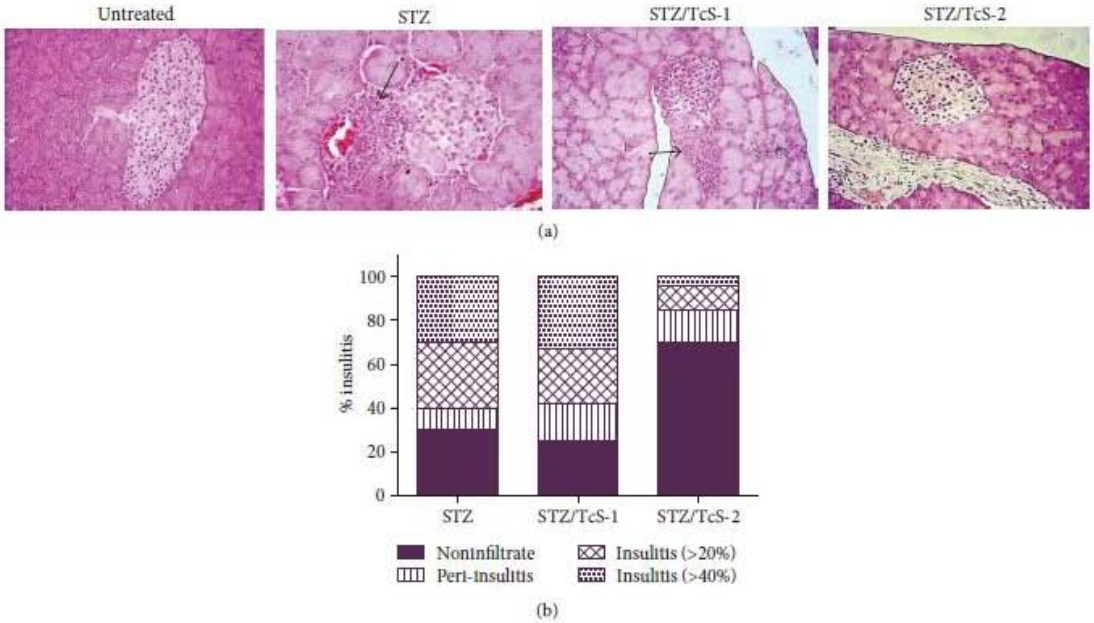


FIGURE 2: Constant TcS exposure reduces insulitis in MLD-STZ-treated mice. T1D-induced mice were treated with TcS for 6 weeks. The animals were sacrificed, and the pancreases were processed, embedded in paraffin, and cut into 5 μm sections. (a) Representative pictures of islets of Langerhans stained with H&E and evaluated microscopically for the presence of insulitis. Arrows show cellular infiltrate in islets of Langerhans. Magnification of 400x. (b) Percentage of insulitis. Score of infiltrated islets. Noninfiltrated: without damage. Peri-insulitis: infiltration only at the periphery of the islets. Insulitis 20%: infiltration of ≥20% or more but less than 40%. Insulitis 40%: infiltrate of ≥40% of islets. The insulitis evaluation shown is representative of 10 mice per group (at least 100 islets were counted).

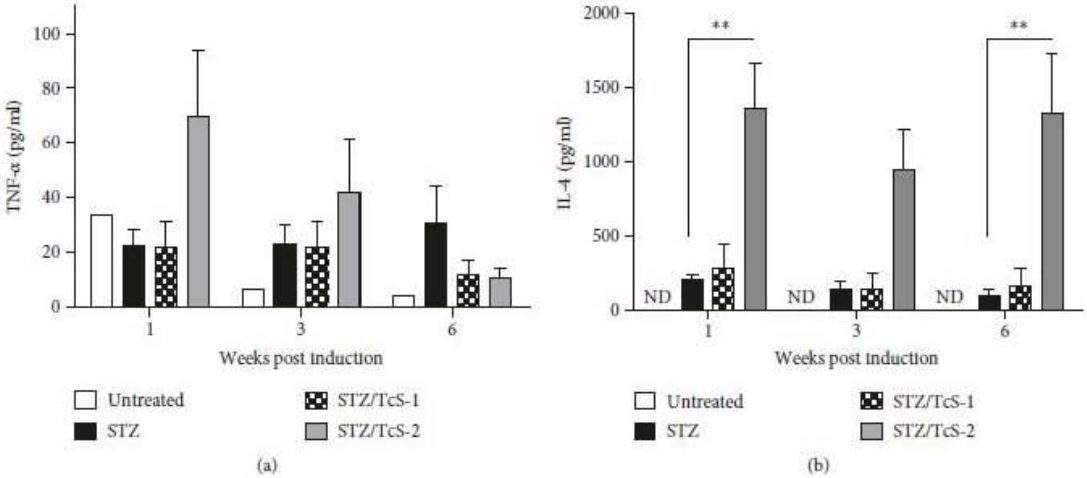


FIGURE 3: Injection of *T. crassiceps* soluble antigen increases IL-4 in serum. Serum was collected from mice at 1, 3, and 6 weeks p.i. (a) TNF-α and (b) IL-4 were detected by ELISA sandwich. ND = nondetected. $^{**}p < 0.01$ by the Mann–Whitney U test. Data are representative of two independent experiments. $N = 4$ mice per group.

3.7. Macrophage Depletion Reverses Helminth-Associated Protection against T1D. To elucidate which cell population was participating in helminth-associated protection against T1D, AAMϕs or CD11b$^+$Gr1$^+$, or both, we infected mice with *T. crassiceps* metacestodes, and 6 weeks later, T1D was induced in the infected mice with MLD-STZ. Later, in the second week post T1D induction, diabetic mice were injected i.p. 3 times a week with 2 mg/mouse of clodronate-loaded liposomes or control PBS-loaded liposomes for 6 weeks post T1D induction (Figure 7(a)). Diabetic mice treated with PBS-

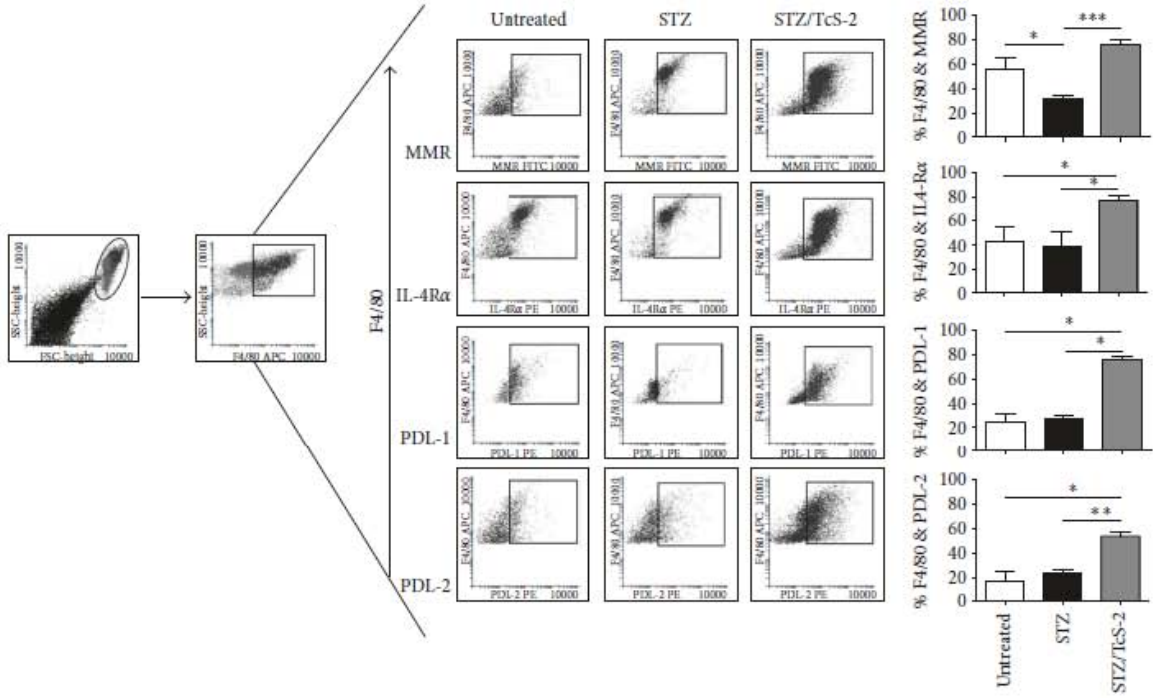
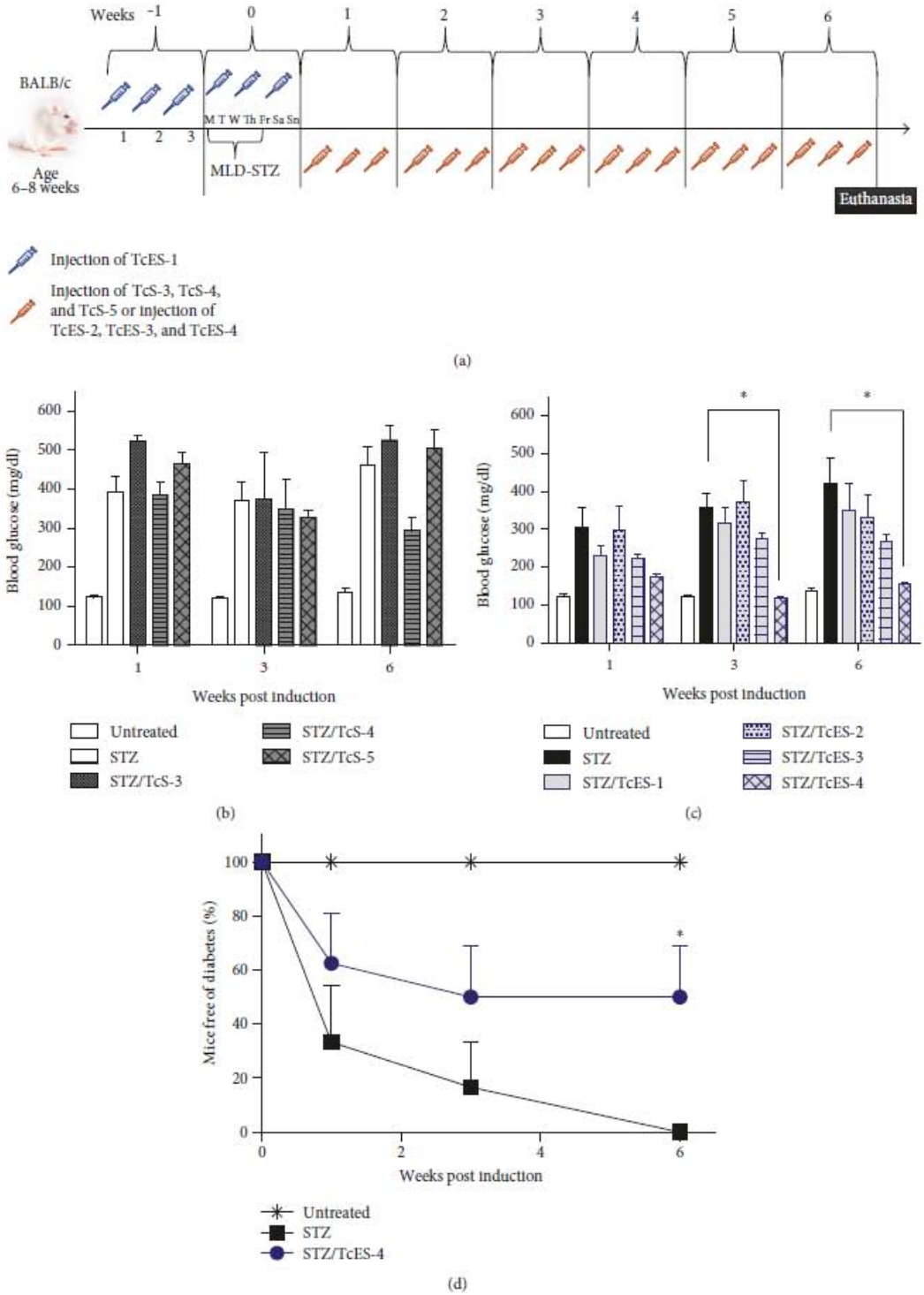


FIGURE 4: AAMϕs appear with constant soluble antigen treatment. PECs were obtained at 6 weeks post induction and stained for AAMϕ markers such as MMR, IL-4Rα, PDL-1, and PDL-2. Its expression was evaluated in F4/80-positive cells by flow cytometry. The data represent at least 2 independent experiments. The data shown are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ by the Mann–Whitney U test, and $^{***}p < 0.001$. $N = 4$ mice per group.

loaded liposomes (STZ PBS) and clodronate-loaded liposomes (STZ Cl) displayed hyperglycemia beginning in the second week post-T1D induction. In contrast, *T. crassiceps*-infected mice receiving control liposomes (STZ/Tc PBS) displayed levels of blood glucose below 200 mg/dl during whole treatment (Figure 7(b)). Interestingly, when *T. crassiceps*-infected mice received clodronate-loaded liposomes (STZ/Tc Cl), the protective effect of this helminth infection was reversed, and mice turned as diabetic as STZ mice, showing hyperglycemia (Figure 7(b)). The incidence of diabetes was also critically affected by clodronate treatment in each group: all STZ PBS mice displayed hyperglycemia by the second week post T1D induction and were considered diabetic, while the STZ Cl mice showed hyperglycemia until the fourth week post T1D induction. In contrast, in the STZ/Tc PBS group, a protective effect was observed, only 50% of these mice had hyperglycemia (>200 mg/dl), and the rest of the animals remained healthy, but when *T. crassiceps*-infected animals received clodronate liposomes (STZ/Tc Cl), all these animals rapidly turned diabetic, with high levels of glucose at the sixth week, and only 15% were free of diabetes (Figure 7(c)). Finally, to gain further insight about whether AAMϕs, $CD11b^{+}Gr1^{+}$ cells, or both were involved in the protection of diabetic mice and whether clodronate-loaded liposomes depleted any other regulatory cell, flow cytometry of peritoneal cells was performed. We observed that STZ mice receiving PBS-loaded liposomes recruited $FSC^{high}SSC^{high}F4/80^{+}$ and $FSC^{high}SSC^{high}CD11b^{+}$ cells in the peritoneal cavity; in contrast, mice receiving clodronate-loaded liposomes displayed a depleted $F4/80^{+}$ cell population but maintained the recruitment of $CD11b^{+}$ cells, demonstrating that clodronate liposomes only depleted macrophages, mostly AAMϕs, and no other populations such as $CD11b^{+}$ cells (Figure 7(d)). Figure 7(e) shows that *T. crassiceps* infection in conjunction with STZ significantly increased the markers MMR and PDL-2 compared with all other groups, whereas infected mice that received clodronate liposomes showed a significant reduction in the expression of these AAMϕ markers.

Next, we look for any differences between two different subpopulations of MDSCs, $CD11b^{+}Ly6C^{+}Ly6G^{-}$ and $CD11b^{+}Ly6G^{+}Ly6C^{-}$, and whether those populations could change their recruitment when we injected clodronate-loaded liposomes in diabetic animals. Flow cytometry of peritoneal cells shows that all groups expressed $CD11b^{+}Ly6C^{+}$ markers at a high percentage except for the untreated group. However, when animals received clodronate liposomes, the percentage of $CD11b^{+}Ly6C^{high}$ cells was significantly higher than in mice receiving PBS liposomes (Figure 7(f)). In contrast, granulocytic cells, $CD11b^{+}Ly6C^{-}Ly6G^{+}$, increased with the injection of clodronate loaded-liposomes; STZ/Tc Cl and STZ/Tc PBS had significant differences compared to mice from the STZ PBS group. Both the STZ/Tc Cl and STZ Cl groups displayed higher percentages of both



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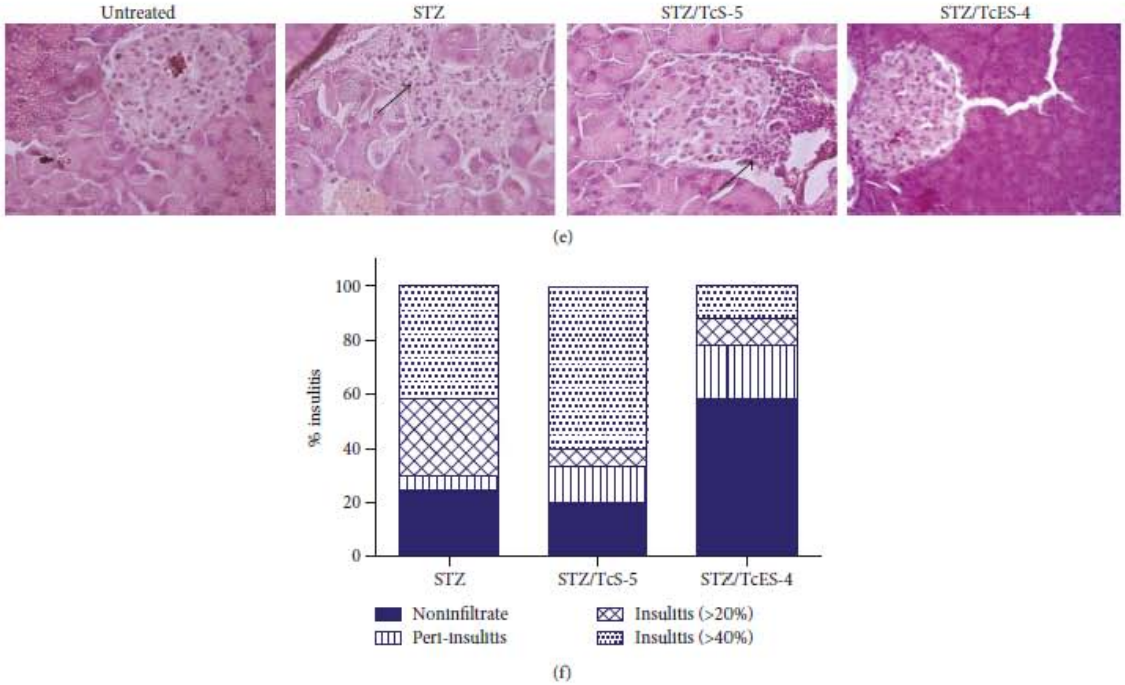


FIGURE 5: Exposure to TcES reduces T1D damage. Mice were injected with soluble antigen (TcS) or excreted/secreted antigen (TcES). Blood glucose levels were measured for 6 weeks after T1D induction. (a) Methodology diagram illustrating injection of *T. crassiceps* antigen (TcS and TcES). (b) Blood glucose of mice treated with different doses of TcS post T1D induction. (c) Glycemia of mice receiving constant and post T1D induction treatment with TcES. (d) Percentage of mice free of diabetes. Mice with glycemia greater than 200 mg/dl were considered diabetic, whereas those with levels of 200 mg/dl or lower were considered free of diabetes. *Differences between STZ and STZ/TcES-4. (e) Histology of pancreas. Arrows represent insulitis and pancreatic islet damage. (f) Insulitis score. Noninfiltrated: without damage. Peri-insulitis: infiltration only at the periphery of the islets. Insulitis 20%: infiltration of ≥20% or more but less of 40%. Insulitis 40%: infiltration of ≥40% of islets. Data are representative of 2 independent experiments. $N = 5$ mice per group. For comparisons between two groups, the Mann–Whitney U test was applied to test differences with nonparametric data. Experiments with multiple groups were tested by the Kruskal-Wallis test followed by Dunn's multiple comparison test. The data shown are the mean ± SEM. $^*p < 0.05$.

$CD11b^+Ly6C^+Ly6G^-$ and $CD11b^+Ly6C^-Ly6G^+$ cells; all these observations suggest that monocytic and/or granulocytic MDSCs could promote an inflammatory response and damage because both groups showed hyperglycemia and incidence of T1D; and furthermore, the recruitment of AAMϕs was less evident in these groups. In contrast, STZ/Tc PBS mice maintained AAMϕ recruitment and displayed both reduced glycemia and reduced incidence of T1D, suggesting that AAMϕs were regulatory cells with an important role in modulating T1D induced by MLD-STZ.

This last experimental design was replicated with animals exposed to TcS, where mice were injected with TcS one week before T1D induction and for 4 weeks afterward. Data shown in Figure 8 indicate that mice exposed to TcS and treated with PBS liposomes (STZ/TcS PBS) displayed reduced hyperglycemia, whereas mice similarly exposed to TcS but receiving clodronate-loaded liposomes (STZ/TcS Cl) are not protected against increasing blood glucose levels, again supporting the idea that AAMϕs may play a central role in T1D protection using helminth-derived molecules.

4. Discussion

Helminths and their products have been suggested as a powerful weapon against many inflammatory diseases, such as type 1 diabetes, arthritis, colitis, encephalomyelitis, Crohn's disease, and asthma [8, 32–34]; such effects are mainly based on the ability of helminths and their antigens to induce strong Th2-biased responses, with increases in cytokines such as IL-4, IL-10, IL-13, and TGF-β [19, 35]. Additionally, they induce regulatory cells, such as AAMϕs, Treg, and MDSCs, which have been linked with decreased inflammatory responses and less tissue damage as well as with wound healing processes [18, 27, 36, 37].

Type 1 diabetes is an autoimmune disease in which insulin-producing β-cells are destroyed by $CD4^+$ and $CD8^+$ T cells and CAMϕs [1, 2]. Studies in animal models such as NOD or MLD-STZ-induced T1D mice have provided evidence for the ability of helminths to reduce inflammatory responses, death of β-cells, and insulitis [7]. In a previous study, we showed that *T. crassiceps* infection was able to

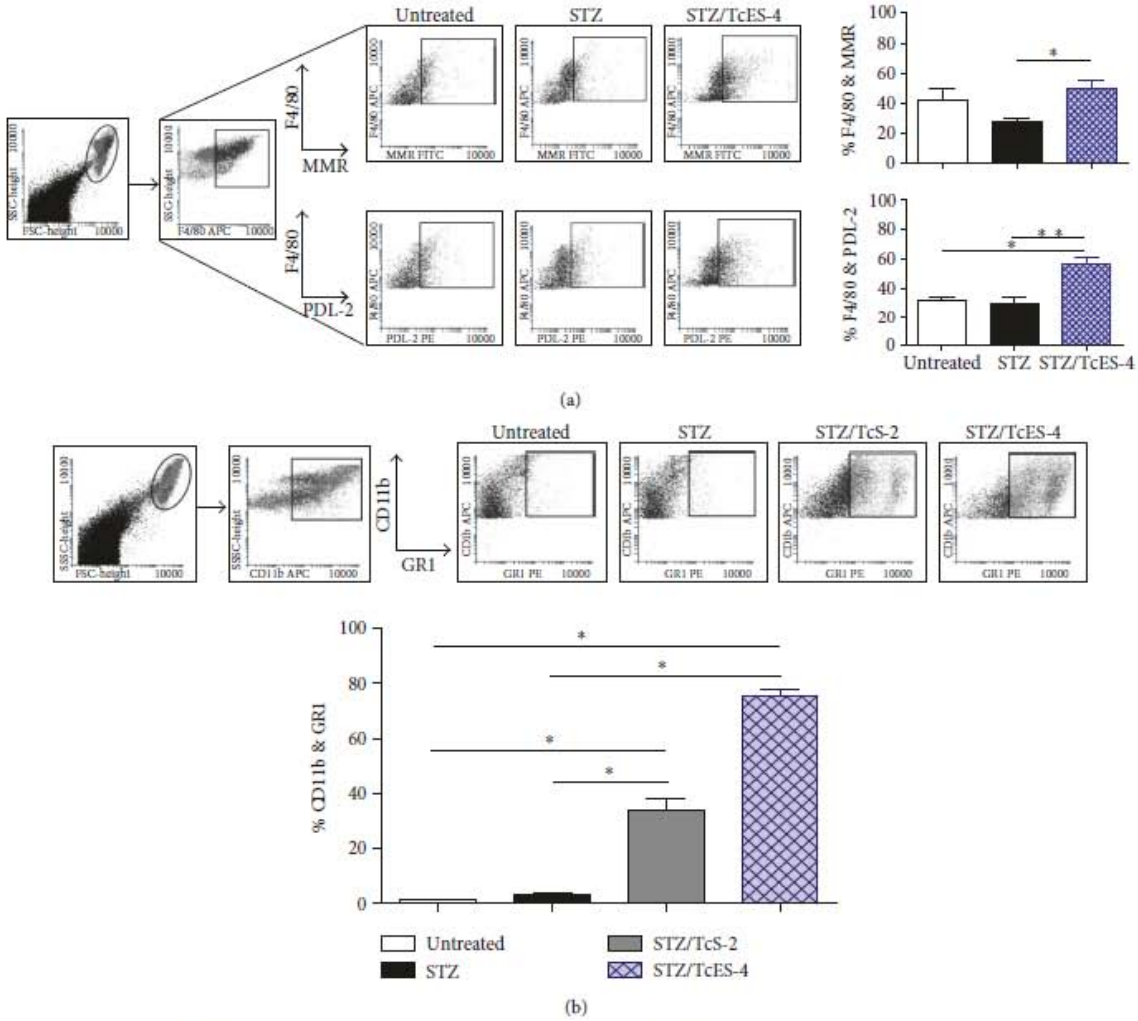
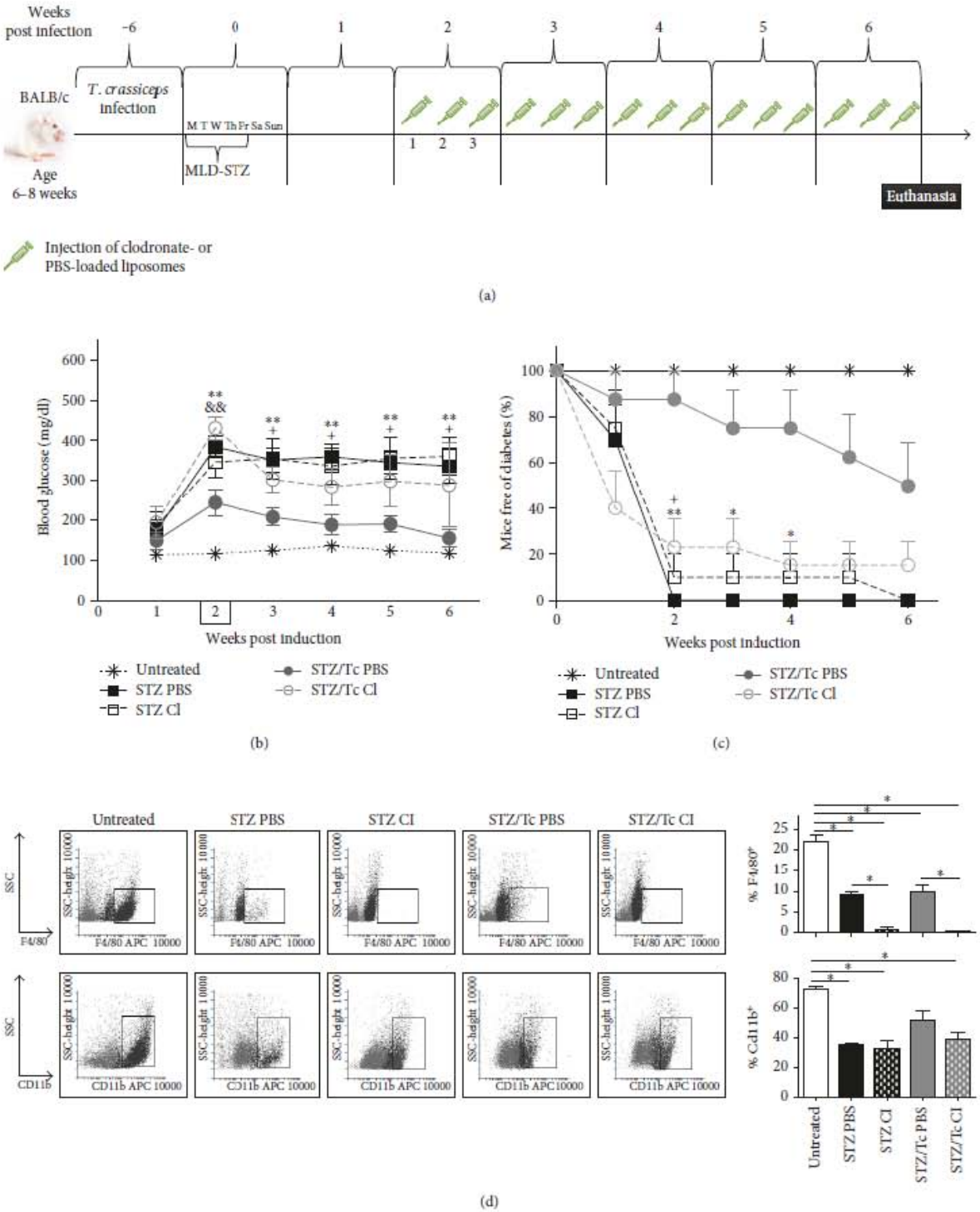


FIGURE 6: Exposure to TcES during T1D increases both AAMϕ and FSC^{high}CD11b^{+} Gr1^{+} populations. (a) PECs were stained for F4/80, MMR, and PDL2 and analyzed in a flow cytometer. (b) PECs were stained for the markers Gr1, CD11c, and CD11b and analyzed by flow cytometry. $^{*}p < 0.05$ by the Mann–Whitney U test. $^{**}p < 0.01$. Data are representative of 2 independent experiments. $N = 4$ mice per group.

reduce hyperglycemia and insulitis associated with strong recruitment of AAMϕs; such data suggest that these cells could be important to prevent pancreatic damage and suppress autoreactive T cells [13]. Additionally, our group showed that *T. crassiceps* infection could decrease inflammatory response and damage by autoreactive cells in colitis and experimental autoimmune encephalomyelitis (EAE) [38, 39]. Our goal in the present work was to investigate whether *T. crassiceps* products (TcES or TcS) would mimic the effect of experimental infection by decreasing inflammatory responses induced by T1D, and we looked for cells involved in this regulatory response. Here, we demonstrate that both treatments, TcS and TcES, can regulate hyperglycemia, insulitis, and the incidence of T1D, but with slight differences. We found that it is important for TcS treatment to begin before MLD-STZ induction of T1D and continue with constant injections of TcS for a protective effect. Otherwise, TcES treatment in diabetic mice was more effective to decrease hyperglycemia at a high dose (200 μg), compared with TcS (50 μg), in a post induction system of MLD-STZ-induced T1D. Differences between the effects of TcS and TcES may be explained by a previous work in which exposure of macrophages to TcES modified their inflammatory response to IFN-γ through the expression of high levels of SHP-1 and SOCS3, which are suppressors of IFN-γ-transducing signaling, but when macrophages were exposed to TcS, these cells did not block IFN-γ signaling [40]. In another research, it was found that *T. crassiceps* E/S antigen induced a tolerogenic phenotype in dendritic cells, stopped their maturation, and downmodulated the expression of costimulatory molecules [29]. Such differences observed in vivo and in vitro between TcS and TcES may be associated with differences in the composition or concentration of biomolecules found in the >50 kDa fraction.



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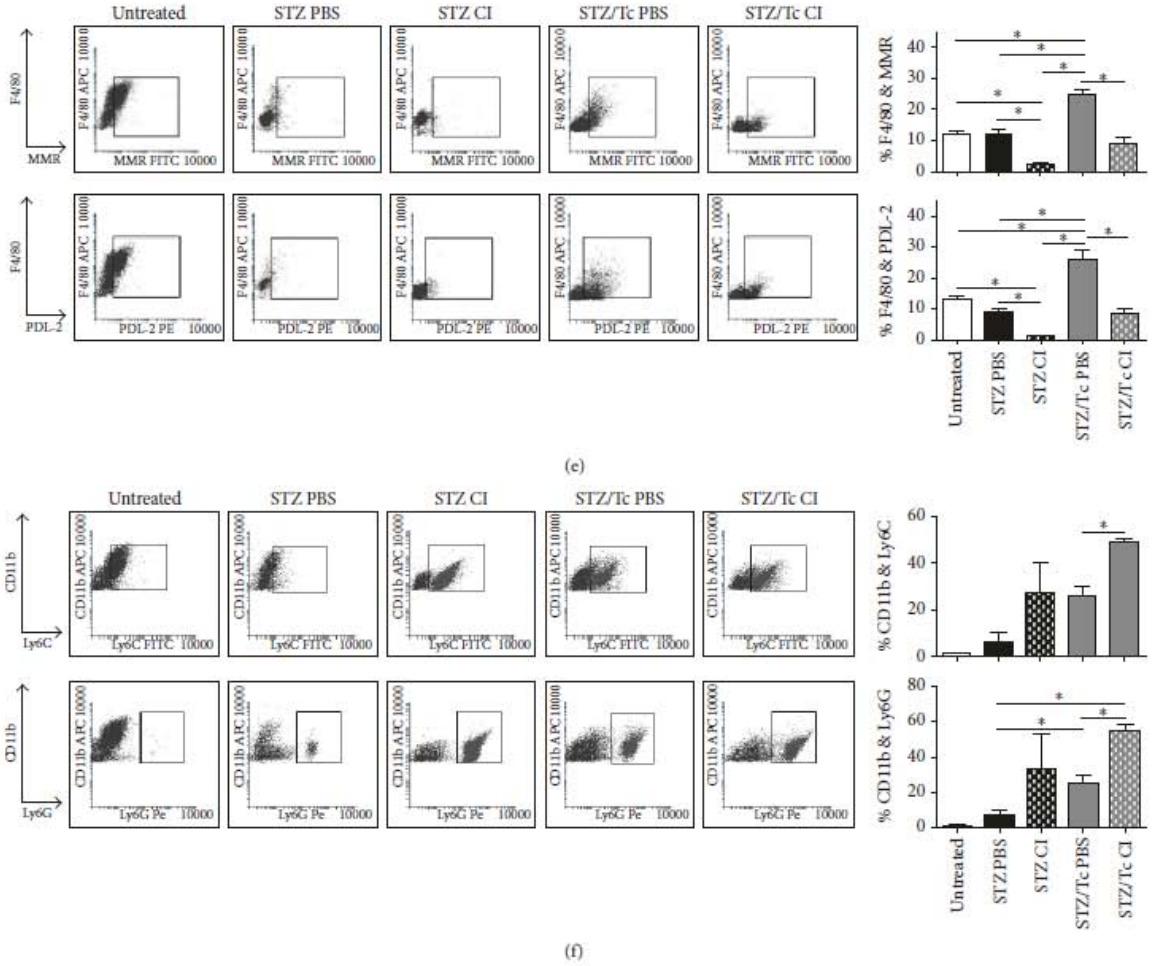

FIGURE 7: Clodronate treatment reveals a role for AAMϕs in T1D protection. Mice were infected i.p. by *T. crassiceps* metacestodes. After the sixth week post infection, mice were induced by MLD-STZ and then were injected with clodronate-loaded liposomes or PBS-loaded liposomes, 3 times for a week during four weeks p.i. T1D. (a) Diagram of experimental design. (b) Blood glucose of mice during 6 weeks post T1D induction. (*Differences between STZ/Tc PBS and STZ PBS. †Differences between the STZ/Tc PBS group and STZ Cl. &&Differences between STZ/Tc PBS and STZ/Tc Cl). (c) Mice free of diabetes. Percent of mice free of diabetes (glycemia higher than 200 mg/dl were considered diabetic). *Differences between STZ and STZ/Tc PBS. (d) Flow cytometry of $FSC^{high}F4/80^{+}$ and $FSC^{high}CD11b^{+}$ peritoneal cells. (e) Flow cytometry of PECs marked for F4/80, MMR, and PDL-2. (f) $CD11b^{+}Ly6C^{+}$ and $CD11bLy6G^{+}$ cells were analyzed in a FACs. For comparisons between two groups, the Mann–Whitney *U* test was applied to test differences with nonparametric data. Experiments with multiple groups were tested by the Kruskal-Wallis test followed by Dunn's multiple comparison test. The data shown are the mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$. Data are representative of 2 independent experiments. $N = 4$ mice per group.

Different studies have demonstrated the ability of several helminth-derived products to reduce T1D development; however, most of them were evaluated as a pretreatment for T1D. For example, Zaccone et al. showed that *S. mansoni* products may prevent the development of diabetes in NOD mice, but only if the treatment was started before the fourth week of age [21]. In other research, Amdare et al. showed that treatment of *B. malayi* ES and soluble (adult and microfilaria) product may protect against T1D, but only if the series of injections were started before T1D induction [41]. More recently, in 2016, Ajendra et al. demonstrated that *L. sigmodontis* antigen (crude worm extract) may protect against T1D in later treatments (after 10 weeks of age) in NOD mice, but only as part of a combined therapy with an intranasal proinsulin dosage [42]. In this context, we believe that TcES could be a promising treatment, considering that its effects do not require additional therapy for protection against T1D. Furthermore, with the reasoning that the diagnosis of T1D in humans is reached late, when most of the β-cells are destroyed, TcES may prevent or delay pancreatic islet destruction after T1D initiation. In line with this idea, another important question is knowing if *T. crassiceps*

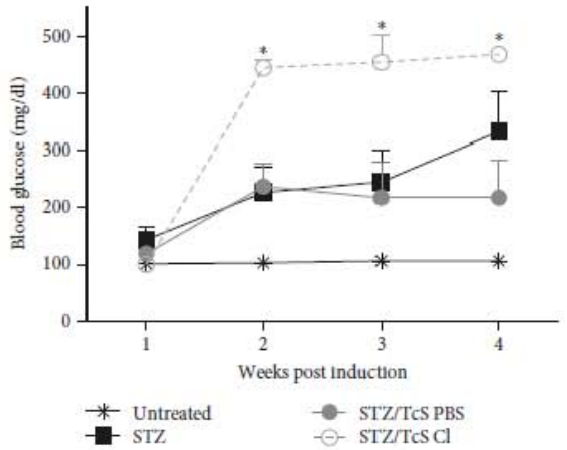

FIGURE 8: Clodronate treatment abrogates protection against T1D in mice receiving TcS. The clodronate-liposome injections followed the same experimental design as the *T. crassiceps* infection process. Mice were injected with TcS 1 week before T1D induction and continue receiving the treatment for 4 weeks post T1D induction. Blood glucose was measured for 4 consecutive weeks. *Differences between STZ/TcS Cl and STZ/TcS PBS. Data are representative by 2 independent experiments, each group with 4 mice. Mean ± SEM. $^{*}p < 0.05$ by the Mann–Whitney *U* test.

antigens can be recognized by human cells; in this regard, Terrazas et.al. showed that human dendritic cells exposed to *T. crassiceps* excreted/secreted antigens displayed a tolerogenic profile [29]. Further studies are needed to prove that TcES may regulate human autoreactive cells as a treatment in T1D in the future.

Another important point about the effect of helminths and their products is their capability to recruit regulatory cells involved in the downregulation of inflammation, as has been demonstrated by several helminth infections (*S. mansoni*, *H. polygyrus*, *B. malayi*, *N. brasiliensis*, *T. spiralis*, *F. hepatica*, and *T. crassiceps)* reducing T1D development; however, all of them were also evaluated as a pretreatment for T1D, and most of them recruited AAMϕs [8, 9, 11, 12]. Some research has also been focused on products of helminths to search for regulatory cells involved in downregulating inflammation. For example, *S. mansoni* products (SEA and SWA) could induce high numbers of AAMϕs and Treg cells [20, 21]. E/S products of *F. hepatica* also increased AAMϕ and Treg populations in diabetic mice, suggesting that both populations are related to protection against diabetes [23]. In our previous research, we found that *T. crassiceps* infection in MLD-STZ-induced T1D mice recruited AAMϕs [13]. Here, we gain knowledge on the role of AAMϕs during *T. crassiceps* antigen treatment (TcS and TcES) in T1D, where we find increased expression of AAMϕ markers such as MMR, PDL-1, and PDL-2 in T1D-protected mice. These data agree with the recent observation that reduction of hyperglycemia in mice infected with *S. mansoni* was Treg independent, while recruitment of AAMϕs was important to the process [43]. In that work, the authors blocked Treg cells by injecting a specific anti-Treg antibody, and hyperglycemia did not improve or even turned worse. However, these same authors did not test a specific role for AAMϕs, whereas in this investigation we show for the first time that AAMϕs are critical for the protective effect of helminth-derived products in T1D development. Moreover, whereas in the *S. mansoni* infection there was only a limited reduction of hyperglycemia and all the animals became diabetic after 3 weeks, here we showed that *T. crassiceps*-derived molecules or even the whole infection was able to reduce the incidence of T1D by 50% for at least 6 weeks. Together, these data indicate that *T. crassiceps* and their derived molecules display more potent anti-inflammatory activity than that observed in other helminthic infections and that such protective effect is highly dependent on the presence of AAMϕs.

Additionally, we observed that TcS and TcES exposure directly upregulated the recruitment of $CD11b^{+}Gr1^{+}$ cells in diabetic mice compared with STZ and untreated mice. MDSCs have garnered increased interest because they can suppress T cell responses. In 2010, Yin et al. [44] demonstrated that adoptive transference of MDSCs in NOD mice significantly decrease diabetes onset as well as pancreatic islet damage, and these authors suggested that MDSCs might mediate anergy of autoreactive T cells and favor the presence of Treg cells. Conversely, in other research, MDSCs were associated with damage, because these cells were favored differentiation of $CD4^{+}$ cells towards a Th17 profile in the pathogenesis of EAE in mice [45].

To elucidate which cells may have a more significant role downregulating T1D in our system, we decide to deplete phagocytic cells by i.p. injections of clodronate-loaded liposomes during both *T. crassiceps* infection and TcS exposure. Previously, it has been demonstrated that clodronate-loaded liposomes only depleted phagocytic cells (mostly macrophages) in our system of peritoneal injections, without affecting dendritic cells and eosinophils [31]. Here, we found that AAMϕs were significantly reduced in STZ/Tc Cl mice, while STZ/Tc PBS recruited higher percentages of AAMϕs. However, we also found that $CD11b^{+}Ly6C^{+}Ly6G^{-}$ and $CD11b^{+}Ly6C^{-}Ly6G^{+}$ populations were increased in mice receiving clodronate liposomes (STZ Cl and STZ/Tc Cl), with significant differences from the other groups. Recently, our group demonstrated that high recruitment of $CD11b^{+}Ly6C^{high}$ in colitic mice was associated with damage and colon inflammation [38]. In line with this previous report, the present study found a similar outcome in T1D mice, with an elevated number of MDSCs (monocytic and granulocytic) in the peritoneal cavity of mice receiving clodronate, but without protection against T1D development; thus, our data strongly suggest that granulocytic and monocytic cells do not participate as a protective cell population in our system. Further studies are needed to elucidate the role of MDSCs in diabetic mice, such as to demonstrate that these cells have suppressive ability, a key feature to name these cells. Instead, a strong role for AAMϕs in the anti-T1D effects of *Taenia* products can be assumed; given that STZ/Tc Cl reversed the protective effect of *T. crassiceps* infection, producing similar hyperglycemia levels to STZ and STZ Cl mice and T1D incidence did reach up to 100%, while the STZ/Tc PBS group was protected up to 50%, displaying hyperglycemia levels

under 200 mg/dl. A reversal of the protective effect of TcS was also observed when mice exposed to TcS received clodronate liposomes. However, clodronate may also affect CAMϕs and thus eliminate a dangerous cell population for T1D development, but given the worsening of the glucose levels, this possible fact may support a more critical role for $CD8^+$ and $CD4^+$ autoimmune cells in tissue damage. Together, these results strongly support the hypothesis that AAMϕs recruited by *T. crassiceps* infection and *T. crassiceps*-derived products are a key population that importantly reduces inflammatory responses associated with T1D development.

5. Conclusion

In conclusion, our findings indicate that exposure to TcS and TcES has a potential protective effect against MLD-STZ-induced T1D development by reducing hyperglycemia and the incidence of this autoimmune disease. Mechanistically, our new data support the hypothesis that AAMϕs recruited by *T. crassiceps*-derived products play a critical role in down-regulating T1D, because when this population is depleted early, the protective effect is abrogated. It is necessary to elucidate the putative signaling pathways triggered by these *T. crassiceps*-derived products to understand more completely the mechanisms associated with their anti-inflammatory and antidiabetic effects.

Conflicts of Interest

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

Acknowledgments

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DISCUSION

Los helmintos pueden modular la respuesta inmune de sus hospederos, modificandola hacia el perfil Th2 e incrementando las poblaciones de Treg, AAMϕS y MDSCs, las cuales ayudan al parásito a permanecer en su hábitat. Se ha comprobado que, algunas infecciones por helmintos pueden ser benéficas en ciertas enfermedades autoinmunes como diabetes, colitis, encefalomielitis [35].

La T1DM se caracteriza por la deficiencia de células β por causa del ataque de células inmunes, provocando hiperglucemia crónica relacionada con falla de varios órganos. Hasta el momento no existe tratamiento ó medicina que sea 100% eficaz en revertir la enfermedad. En el caso específico con helmintos parásitos se ha demostrado que pueden regular en cierta forma el desarrollo de la T1DM en modelos experimentales. Por ejemplo, en ratones NOD, la infección con *S. mansoni* provoco menor inflamación en páncreas, disminuyeron la hiperglucemia e incidencia y encontraron Treg, como posibles células responsables de la regulación de la T1DM [29]. En el caso de *T. crassiceps* se demostró que la infección puede disminuir la colitis y la encefalomielitis en modelos experimentales en ratones [36, 37]. Además, en un trabajo previo, comprobamos que la infección con *T. crassiceps* en ratones con T1DM, puede ser reguladora, disminuyendo el infiltrado inflamatorio del páncreas, la glucemia y consecutivamente, observándose menor incidencia de la enfermedad. A diferencia de otros parásitos que también pueden regular las enfermedades autoinmunes, la infección con *T. crassiceps* no repercute en el daño en otros órganos y puede permanecer en su hospedero por largo tiempo, además de que la infección no induce la población de Treg [24].

El objetivo principal del presente trabajo fue demostrar sí la administración de los antígenos de *T. crassiceps* (TcS o TcES) pueden modular la respuesta inmune de su hospedero y por tanto, reducir los signos y síntomas de la T1DM. También, encontrar que tratamiento resultó más efectivo e investigar que células podrían estar involucradas en la regulación.

Encontramos que, la administración del antígeno TcS antes de la inducción de la T1DM y de manera constante durante las 6 semanas posteriores, puede disminuir la glucemia y, por tanto, 50% de los ratones presentaron glucemias <200mg/dl, comparados con los del grupo STZ, donde

todos desarrollaron diabetes. Lo importante de este punto, fue demostrar que la regulación solo se presentaba si la administración del antígeno era de forma constante, ya que al administrar el TcS por únicamente dos semanas (STZ/TcS-1), la incidencia de los animales con hiperglucemias se incrementó en >80%. En consecuencia, cuando revisamos infiltrado en páncreas, se observó menor daño e infiltrado en los islotes del grupo STZ/TcS2 comparados con el grupo STZ/TcS1 y STZ.

Con respecto al antígeno TcES, encontramos que, el mejor tratamiento fue la administración posterior a la inducción de la diabetes en una dosis de 200µg, ya que se disminuyeron las glucemias considerablemente y solo el 50% de los animales desarrollaron hiperglucemias, en comparación con el grupo STZ, con un 100% de animales diabéticos. Una de las diferencias entre ambos antígenos, fue observar que la administración del TcS posterior a la inducción de la T1D no pudo disminuir la hiperglucemia de los ratones; pero el tratamiento con el TcES si tuvo un efecto protector, posterior a la inducción de la diabetes. Anteriormente, Becerra-Diaz et al., (2014) encontró que la exposición de TcES en macrófagos in vitro modifica su respuesta inflamatoria por la expresión alta de SHP-1 y SOCS3, las cuales son moléculas supresoras de la señalización de IFN-γ, mientras que, cuando los macrófagos fueron expuestos a TcS, las células no bloquearon la vía de señalización de IFN-γ [38]. En otra investigación, se demostró que el TcES induce un fenotipo tolerógenico en las células dendríticas en cultivo in vitro, provocando que se detenga su maduración y disminuyendo la expresión de moléculas coestimuladoras [39]. Estas diferencias que se observaron en trabajos previos y en el presente trabajo nos lleva a plantear la siguiente hipótesis, posiblemente las diferencias entre ambos antígenos está asociada con la composición o concentración de biomoléculas que se encuentran en la fracción de >50kDa, y por tanto el TcES es más efectivo protegiendo de la inflamación que el tratamiento con TcS.

Diferentes estudios han demostrado la habilidad de ciertos productos de helmintos de regular el desarrollo de la T1D; sin embargo, en muchos de ellos se evalúa su eficacia como un pre-tratamiento. Por ejemplo en *S. mansoni*, tanto el antígeno extraido de huevos, como el soluble de parásitos adultos, resulto ser protector para la T1DM en ratones NOD, pero solo si se comenzaba el tratamiento antes de que la enfermedad se desarrolle (es decir, en la semana 4)

[30]. En otra investigación, Amdare *et. al*., (2015) mostraron que el tratamiento con *B. malayi* (ES y soluble) pueden proteger de la T1DM, pero únicamente sí se comienza antes de que se presenten hiperglucemias [40]. En el 2016, se demostró que el tratamiento con antígeno de *L. sigmodontis* (extracto crudo) puede proteger de la T1DM en ratones NOD, a pesar de iniciar en una etapa tardía (posterior a la semana 10) siempre y cuando se combine el tratamiento con dosis intranasales de proinsulina [41]. En este contexto, comparando los últimos estudios con el presente trabajo, el tratamiento con TcES resulta ser un mejor tratamiento, considerando que no requiere una terapia adicional y teniendo en cuenta que, en humanos, el diagnóstico de la T1DM se presenta en una etapa tardía donde muchas células β ya fueron destruidas. El tratamiento con el TcES podría prevenir o retrasar el daño de la T1DM. Además, basado en un estudio con células dendríticas de humano, se demostró que la exposición con TcES, las modifica hacia un perfil tolerógenico. Es decir, que el TcES es reconocido por células humanas llevándolas hacia un perfil no inflamatorio [39]. Se requieren estudios posteriores para comprobar que el TcES puede regular las células autoreactivas de humanos como un futuro tratamiento para la T1DM.

Un punto importante de las infecciones parasitarias es su capacidad de reclutar células reguladoras involucradas en disminuir la inflamación. Se conoce que los AAMϕs pueden liberar citocinas reguladoras como IL-10, que impiden la liberación de citocinas proinflamatorias. Además, los AAMϕs se han relacionado con impedir el daño de tejidos y en la restauración, ya que liberan arginina-1 que, en el metabolismo de la arginasa, producen colágeno y poliaminas, importantes moléculas relacionadas con la restauración [13, 14]. Previamente, habíamos comprobado que los ratones infectados con *T. crassiceps* y T1DM reclutan de forma importante la población de AAMϕs en peritoneo [24]. En este trabajo, comprobamos la presencia de AAMϕs en el tratamiento con el TcES y TcS en ratones inducidos con T1DM.

Adicionalmente, comprobamos que el tratamiento con TcS o TcES en ratones con diabetes, incrementa de manera importante la población CD11b+Gr1+, posiblemente MDSCs. En el 2010, Yin *et al*., demostró que la transferencia adoptiva de MDSCs en ratones NOD significativamente disminuye el desarrollo de la diabetes, también la insulitis y favorece la presencia de Treg [23].

De forma contraria, en otro trabajo asociaron la presencia de MDSCs con daño, así como la diferenciación de los linfocitos T CD4+ a Th17 en la patogénesis de la encefalomielitis experimental [42].

Para elucidar cuales son las células encargadas en disminuir la T1DM en este trabajo, decidimos eliminar los AAMϕs por medio de inyecciones i.p de liposomas con clodronato en ratones. El clodronato, mata a las células fagociticas induciendo apoptosis en las mismas una vez liberado del liposoma. En los resultados, comprobamos por medio de citometría de flujo, que los liposomas con clodronato eliminan de manera específica a los AAMϕs, pues la población F4/80+ es menor que los animales STZ/Tc PBS; mientras la población CD11b+ permanece de forma importante en el grupo STZ/Tc Cl, comprobando que solo se eliminan los macrófagos mas no las células MDSCs con el tratamiento con clodronato. Otro punto importante fue observar que como ya sabíamos, los ratones STZ/Tc PBS pueden reducir la insulitis y controlar las glucemias, disminuyendo la incidencia de animales diabéticos; pero cuando los animales fueron inyectados con liposomas con clodronato, la protección no aparece y comienzan a elevarse las glucemias, al punto que en la semana 6 todos los animales se consideraron diabeticos (con hiperglucemias >200mg/dl). Observamos también que, la población de MDSCs no se eliminaron con las inyecciones de liposomas con clodronato, incluso se incrementó la población en los animales con hiperglucemias, lo que nos hace creer que estas células se relacionan con el desarrollo de la T1DM, incrementando la inflamación y que por tanto, son los AAMϕs, las células importantes en la regulación de la T1DM.

El mismo experimento de liposomas se repitió pero con el tratamiento con el TcS de forma constante. Al igual que con la infección, la inyección del antígeno de *T. crassiceps* también puede regular la diabetes y en los animales con inyecciones de liposomas con clodronato, el efecto regulador se revierte y se observan glucemias superiores a los 200 mg/dl. Por tanto, podemos sugerir que tanto en la infección como en el tratamiento con los antígenos de *T. crassiceps*, los AAMϕs son las células protagonistas en impedir el desarrollo de la diabetes.

Por último, se observaron células Ly6C y Ly6G, en los tratamientos con liposomas tanto en la infección como en el antígeno soluble. Observamos que se incrementan tanto la población Ly6C

como Ly6G en la infección con *T. crassiceps*, pero el tratamiento con liposomas con clodronato provoca que incrementen de forma considerable la expresión de ambas poblaciones. En cuanto al TcS y liposomas, se incrementa de forma importante solo la población Ly6G pero solo si se inyecta a los animales con liposomas con clodronato. Hasta este momento se desconoce el papel que pudieran jugar estas poblaciones tanto en la infección o tratamiento de los antígenos de *T. crassiceps* como en la diabetes. Nosotros sugerimos que estarían participando en la inflamación pero se requiere de más investigaciones que eluciden esta cuestión.

Esta es la primera vez que se comparan antígenos de *T. crassiceps* con la modulación de la respuesta inmune en animales con una enfermedad inflamatoria, como la diabetes tipo 1. Pero aún quedan dudas que tenemos que resolver y que serían de gran importancia científica. En primera instancia conocer un poco más sobre el antígeno TcES, ya que resultó ser protector inclusive posterior a la inducción de la diabetes, y esto nos lleva a pensar que presenta mayor cantidad de moléculas que inducen tanto la respuesta Th2 como células reguladoras. Además de conocer que papel estarían jugando las MDSCs en la diabetes tipo 1. Rebuscar un poco más acerca de los AAMϕs y si están células provenientes del antígeno o infección de *T. crassiceps* y transferidas a ratones con diabetes pueden modular la respuesta inmune.

CONCLUSIONES

- Se demostró que la administración del TcS y TcES provenientes de *T. crassiceps* pueden modificar la respuesta inmune de sus hospederos y son protectores en la T1DM.

- La administración del antígeno soluble tiene un efecto protector a una concentración de 50 µg, siempre y cuando sea constante, y previo a la inducción de la T1DM, ya que comprobamos que si se deja de administrar el tratamiento, se pierde su efecto protector.

- El tratamiento con TcES de *T. crassiceps* tuvo un efecto regulador de la T1DM incluso cuando inicio después de la inducción de la diabetes (a la concentración de 200 µg). Observamos una disminución en la hiperglucemia e insulitis.

- En ambos tratamientos hubo un incremento en la población de AAMϕs y de células CD11b+, que son MDSCs.

- Se incrementaron las poblaciones $CD11b^{+}Ly6C^{+}$ y $CD11b^{+}Ly6G^{+}$, relacionadas al incremento de daño e incidencia de la T1DM.

- La regulación de la T1DM fue dependiente de la presencia de los AAMϕs en la infección y administración de antígenos de *T. crassiceps.*

- Los AAMϕs son las células principales en la regulación por medio del tratamiento del antígeno e infección con *T. crassiceps* en el modelo de T1DM por MLD-STZ, disminuyendo la inflamación, hiperglucemia y la incidencia de la enfermedad.

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Anexo 1.

Se adjuntaron dos artículos, que fueron parte del proyecto.

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Research Article

Taenia crassiceps Infection Attenuates Multiple Low-Dose Streptozotocin-Induced Diabetes

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Taenia crassiceps, like other helminths, can exert regulatory effects on the immune system of its host. This study investigates the effect of chronic *T. crassiceps* infection on the outcome of Multiple Low Dose Streptozotocin-Induced Diabetes (MLDS). Healthy or previously *T. crassiceps*-infected mice received MLDS and type 1 diabetes (T1D) symptoms were evaluated for 6 weeks following the induction of MLDS. *T. crassiceps*-infected mice displayed lower blood glucose levels throughout the study. A significantly lower percentage of *T. crassiceps*-infected mice (40%) developed T1D compared to the uninfected group (100%). Insulitis was remarkably absent in *T. crassiceps*-infected mice, which had normal pancreatic insulin content, whereas uninfected mice showed a dramatic reduction in pancreatic insulin. Infected mice that received MLDS did not show an increase in their regulatory T cell population, however, they had a greater number of alternatively activated macrophages, higher levels of the cytokine IL-4, and lower levels of TNF-α. Therefore, infection with *T. crassiceps* causes an immunomodulation that modifies the incidence and development of MLDS-induced autoimmune diabetes.

1. Introduction

Parasitic helminths are a highly diverse group of organisms that display different morphologies, accessory structures, sexual and feeding behaviors and life cycle stages. Helminths distribute themselves across a variety of niches inside their hosts where they can cause a multiplicity of diseases. Helminth parasites also appear to follow varied and complicated oral and cutaneous routes of infection within host tissues. Surprisingly, despite these differences in features and behavior, helminths share a unique ability to exert profound regulatory effects on the immune systems of their hosts. One of the first observations made concerning helminth infection was the elicitation of a strong Th2-biased immune response [1, 2]. In the last few years, new regulatory has been identified to play a role in helminth infection [2, 3]. One of these mechanisms includes the induction of regulatory T cells (Tregs), which are now known to be involved in pathogen susceptibility and the control of inflammation in helminth infections caused by *Litomosoides sigmodontis* [4], *Trichuris muris* [5], *Brugia malayi* [6], *Trichinella spiralis* [7], and *Heligmosomoides polygyrus* [8]. Dendritic cells (DCs) have also been reported to be affected by helminth-derived products [3, 9] and, more recently, a new population of macrophages called alternatively activated macrophages (AAMsϕ) has been consistently observed in several worm infection models [10, 11].

There are a series of epidemiological and experimental studies supporting the idea that helminth infections can induce a protective effect against the development of both autoimmune and allergic diseases [12]. The "hygiene hypothesis" was the first to suggest that the increase in the prevalence of allergies and asthma in developed countries might be linked to the reduction in infections with parasitic and bacterial pathogens. Thus, parasitic infection might somehow "educate" the immune system to avoid exacerbated

inflammatory responses [13, 14]. Type 1 diabetes (T1D) is an autoimmune disease that has increased in prevalence over the last several years in developed countries and is caused by the selective destruction of insulin-producing β cells located in pancreatic Langerhans' islets by autoantigen-reactive inflammatory T cells. When the majority of β cells are destroyed, the pancreas' ability to secrete insulin in response to blood glucose levels is impaired, resulting in a disruption of glucose homeostasis. Previous studies have shown that the MLDS-induced diabetes model is a useful tool for understanding the basic mechanisms associated with the origin and modulation of induced T1D. T1D has been correlated predominantly with genetic background as well as proinflammatory cytokine profiles for TNF-α, IL-12 and IFN-γ [15, 16]. For example, C57BL/6 mice are more susceptible to developing MLDS-induced T1D than mice lacking STAT-4, a transcription factor that is essential for IL-12 signaling. STAT-4 deficient mice have a delayed onset of MLDS-induced T1D and show a milder form of the disease [17].

Taenia crassiceps is a cestode parasite that is useful in infection model systems for cysticercosis [18]. Infection of inbred mice with *T. crassiceps* induces a strong Th2-like immune response that is similar to the response elicited by infection with other helminths [19]. In addition to this Th2-like response, *T. crassiceps* infection is associated with a series of immunomodulatory events including the induction of AAMϕ [20] and the inhibition of T cell proliferative responses to bystander and polyclonal stimuli [2]. We and others have found that infection with *T. crassiceps* alters the immune response to and susceptibility to concomitant pathogens such as *Trypanosoma cruzi* [21], vaccinia virus [22], or *Leishmania* [18], and also reduces the efficacy of vaccination [23]. Thus, it is clear that *T. crassiceps* infection is able to modify immune responses to concomitant pathogens.

The purpose of this study is to determine whether the immune modulation that is induced by *T. crassiceps* infection might affect the outcome of Multiple Low Dose Streptozotocin-Induced Diabetes (MLDS). To address this question, we compared the course of MLDS development in both healthy and *T. crassiceps*-infected mice. Our data suggest that *T. crassiceps* infection might modify the incidence and development of MLDS-induced autoimmune diabetes.

2. Materials and Methods

2.1. Mice. Six- to eight-week-old male BALB/cAnN mice and C57BL/6NHsd mice were purchased from Harlan Laboratories (México) 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. Parasites and Infection Protocols. Metacestodes of *Taenia crassiceps* (ORF) were harvested in sterile conditions from the peritoneal cavity of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) and used for mouse infection. Male BALB/c and C57BL/6 mice were infected with an intraperitoneal (i.p.) injection of 20 small, nonbudding cysticerci of *T. crassiceps* resuspended in 0.3 mL of PBS.

2.3. Multiple Low-Dose Streptozotocin-Induced Diabetes (MLDS). Mice infected with *T. crassiceps* for 6 weeks or uninfected controls received daily intraperitoneal injections of 40 mg/kg streptozotocin (Sigma-Aldrich; dissolved in 0.1 M sodium citrate, pH 4.5) for 5 consecutive days. Blood glucose was measured in animals that were fasted for 6 hours by an Accu-chek Advantage glucometer (Roche Diagnostics) once per week over a 6-week period. Untreated mice were included as controls. Animals were considered diabetic when fasting blood glucose was greater than means ± 2 SD on two consecutive tests.

2.4. Intraperitoneal Glucose Tolerance Test. Uninfected and *T. crassiceps*-infected mice were subjected to an intraperitoneal glucose tolerance test in order to establish the effects of the MLDS-induced diabetes model on glucose metabolism (n = 6–10 for each group). Uninfected and infected MLDS-treated mice were fasted for 6 hours prior to sample collection. A basal blood sample (0 min) was collected by tail-snip and plasma glucose was evaluated using an Accu-chek Advantage glucometer. Mice were then injected i.p. with filtered d-glucose (1.5 mg/kg). Glucose levels were evaluated again at 30-, 60-, and 120-minute time points.

2.5. Histology. Pancreata from C57Bl/6 and BALB/c mice were collected 6 weeks after the induction of diabetes. Tissue was processed and embedded in paraffin, and 5-μm sections were cut for histological analysis. Thin sections were stained with hematoxylin-eosin and evaluated microscopically for the presence of insulitis using the following scoring system: grade 0, normal; grade 1, minor peri-islet cell infiltration; grade 2, moderate infiltration (<50% of islet area); grade 3, severe infiltration (>50% of islet area) with damage to islet architecture.

2.6. Immunohistochemistry. Immunoperoxidase staining was performed on 5-μm paraffin sections using an avidin-biotin complex system, the Insulin Ab-6 (INS04 + INS05) mouse monoclonal antibody, and the commercial kit Dako EnVision + System-HRP (DAB). Sections were counterstained with hematoxylin. Images were captured using AxioVision Rel 4.6An and an AxioCam ICc3 connected to a Zeiss Microscope recorded the images.

2.7. Cytokine ELISAs. Peripheral blood was collected from tail snips once a week over a 6-week period. Serum IL-4 and TNF-α levels were measured by sandwich ELISA using commercial kits purchased from Peprotech (Rocky Hill, NJ, USA).

2.8. Isolation of Peritoneal Macrophages. BALB/c and C57BL/6 were sacrificed 6 weeks after induction diabetes. Peritoneal exudate cells (PECs) from mice STZ, STZ/Tc, and

Normal mice were obtained using 5 mL of ice-cold sterile PBS and the red blood cells were lysed by resuspending the cells in Boyle's solution. Following two washes, the viable cells were counted by trypan blue exclusion with a Neubauer hemocytometer. PECs were adjusted to 5×10^6/mL in RPMI medium and then cultured in six-well plates (Costar, Cambridge, Mass). After 2 hours at 37°C and in 5% CO_2, nonadherent cells were removed by washing them with warm supplemented RPMI medium. Peritoneal macrophages were aseptically removed and processed for RNA extraction using the TRIzol reagent (Invitrogen, Carlsbad, CA).

2.9. Analysis of Cell Surface Markers in Macrophages. The Fc receptors on peritoneal macrophages were blocked with anti-mouse CD16/CD32 (Biolegend, CA, USA) and then stained with an APC-conjugated monoclonal antibody against F4/80 (Biolegend, CA, USA), PE-conjugated antibodies against PD-L1 and PD-L2, FITC-conjugated antibodies against CD23, or Alexafluor-conjugated antimannose receptor antibody (all obtained from Biolegend). The stained cells were analyzed on a FACsCalibur flow cytometer using Cell Quest software (Becton Dickinson).

2.10. T-Reg Cells Detection. Limph nodes were macerated individually using frosted glass slides. The staining Treg cells were according to the manufacturer instruction (Mouse Treg Flow Kit, Biolegend).

2.11. Reverse Transcriptase-PCR. Total RNA was extracted from purified peritoneal macrophages obtained from BALB/c and C57BL/6. mRNA transcripts in peritoneal macrophages was determined by reverse transcription (RT)-PCR. The RNA was quantified and 3 mg of RNA were reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen) and an oligo dT primer, as recommended by the manufacturer. Once cDNA was obtained, conventional PCR was performed. The PCR reactions contained (in a 25 mL final volume) 5 × PCR buffer blue, 10 mM dNTP, 40 nM each forward and reverse primer, 1 unit of Taq DNA polymerase (Sacace Biotechnologies, Italy), and 2 mL of the cDNA. The program used for the amplification of each gene was an initial denaturation at 95 8°C for 5 minutes, 35 cycles of 95 8°C for 40 seconds, the indicated melting temperature for 50 seconds and 72 8°C for 40 seconds and a final extension step of 72 8°C for 4 minutes. All reactions were carried out in a thermal cycler (Corbett Research, Australia). Finally, to observe the amplified products, a 1.5% agarose gel was prepared and samples were loaded with blue juice buffer containing SYBR Green (Invitrogen). The gels were visualized using a Fujifilm FLA 5000 scanner (Fuji, Japan) with FLA 5000 image reader V2.1 software to capture the shown images.

2.12. Statistical Analysis. One-tail Student t-test (glycemia, cytokine, and glucose tolerance test) was applied. $P < .05$ was considered statistically significant.

3. Results

3.1. Taenia crassiceps Infection Modulates Hyperglycemia and Diabetes Incidence by Multiple Low Dose Streptozotocin-Induced Diabetes. This study investigates whether *T. crassiceps* infection modifies the onset and development of MLDS-induced diabetes. Six weeks after infection with *T. crassiceps*, male BALB/c and C57BL/6 mice received MLDS (40 mg/kg) for 5 consecutive days. Blood glucose levels were analyzed each week for six weeks following the treatment with MLDS. Uninfected BALB/c mice became hyperglycemic one week post-MLDS injection (Figure 1(a)). By the second week following the induction of diabetes 100% of the uninfected mice were diabetic and remained diabetic until the end of the experimental period. In contrast, *T. crassiceps*-infected BALB/c mice displayed significantly lower blood glucose levels throughout the six-week period following MLDS injection compared to uninfected mice (Figure 1(a)). Interestingly, the onset of diabetes (as determined by glucose levels) was also different between uninfected and *T. crassiceps*-infected mice. The onset of diabetes in *T. crassiceps*-infected BALB/c mice occurred 2 weeks after MLDS induction; whereas the onset of diabetes occurred within the first week following MLDS-induction in uninfected mice. Additionally, only 40% of *T. crassiceps*-infected mice developed hyperglycemia compared to 100% in uninfected mice (Figure 1(b); $P < .05$). Interestingly, *T. crassiceps*-infected mice had normal glucose tolerance test values (Figure 1(c)). In contrast, uninfected mice could not down-modulate the hyperglycemia until 2 hours after the glucose tolerance test was administered.

A different trend was observed in C57BL/6 mice. C57BL/6 mice are known to be more susceptible to MLDS-induced diabetes [24] and are more resistant to *T. crassiceps* infection [25, 26]. C57BL/6 mice developed higher levels of hyperglycemia than BALB/c mice, but these high glucose levels were controlled after 4 weeks by *T. crassiceps* infection (Figure 2(a)). Shortly after MLDS-induction, 80% of *T. crassiceps*-infected C57BL/6 mice were diabetic (Figure 2(b)). However, by 4 weeks following MLDS-induction infected C57BL/6 mice began to recover from the hyperglycemia. Only 20% of *T. crassiceps*-infected C57BL/6 mice were diabetic at the end of this experiment. MLDS treatment caused significant elevations in glucose levels during an intraperitoneal glucose tolerance test in uninfected mice, but mice carrying *T. crassiceps* displayed normal glucose tolerance test values (Figure 2(c)).

3.2. Lack of Insulitis in T. crassiceps Infected Mice Treated with MLDS. It is known that MLDS stimulates the recruitment of leukocytes to pancreatic islets, resulting in insulitis [15]. Pancreata were harvested at the end of the 6-week experimental period for histopathological analysis. Figure 3 illustrates islet histopathology and the development of insulitis in uninfected and *T. crassiceps*-infected BALB/c and C57BL/6 mice. A significant number of infiltrating leukocytes and the presence of insulitis was observed in the pancreata from uninfected mice (Figures 3(a) and 3(b)), which was associated with loss of islet architecture in some cases. Conversely,

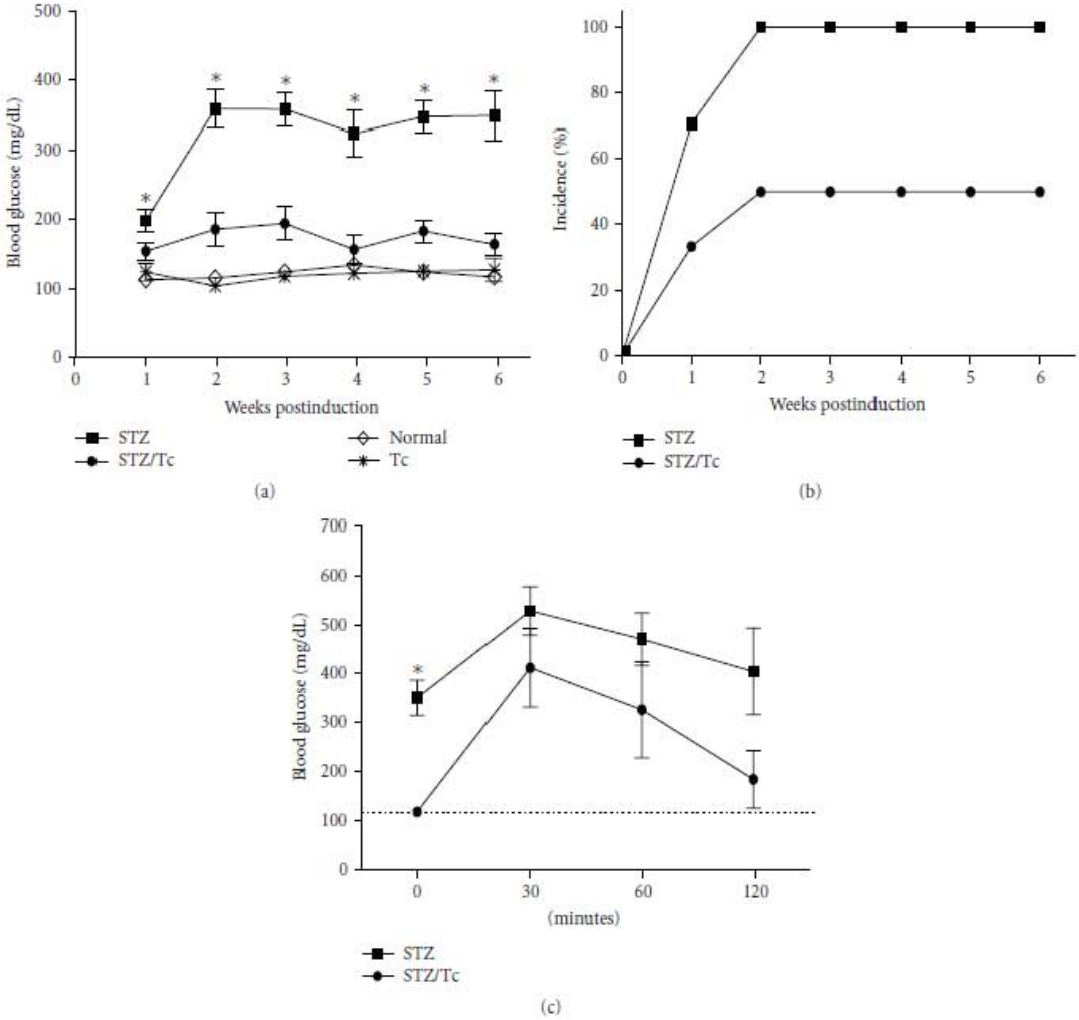

FIGURE 1: Hyperglycemia and diabetes incidence is modified by *T. crassiceps* infection in BALB/c mice. (a) Blood glucose levels throughout the MLDS protocol for uninfected BALB/c mice and BALB/c mice infected with *T. crassiceps* for 6 weeks. (b) Percent incidence of diabetes between uninfected and *T. crassiceps*-infected BALB/c mice. (c) Intraperitoneal glucose tolerance test for MLDS-treated uninfected and *T. crassiceps*-infected mice. Dotted line indicates normal glucose values. $^*P < .05$, $n = 12$.

the islet histopathology from *T. crassiceps*-infected mice was devoid of both cellular infiltrates and insulitis (Figures 3(a) and 3(b)). Similar results were observed in the pancreata from BALB/c and C57BL/6 mice.

3.3. Immunohistochemistry of Insulin in MLDS-Treated Mice. To determine whether islet cells from either uninfected or *T. crassiceps*-infected mice are able to produce insulin, we performed specific immunostaining of pancreatic tissue using an anti-insulin antibody. MLDS-treated *T. crassiceps* infected mice demonstrated strong insulin staining in their pancreatic islets as compared with normal (untreated and uninfected) mice (Figure 4(a)). In contrast, MLDS-treated uninfected mice had weak insulin staining, suggesting a substantial loss of insulin granules in islet β-cells. A similar result was obtained from C57BL/6 mouse tissues (Figure 4(b)).

3.4. T. crassiceps Infection Increases IL-4 Levels in MLDS-Treated Mice. To analyze whether the inhibition of diabetes in helminth-infected MLDS-treated mice was due to an alteration in the Th1/Th2 balance, cytokine levels from the sera of uninfected and infected mice were measured. The Th1-associated cytokines IFN-γ and TNF-α have been reported to accompany the development of diabetes in both NOD and MLDS-induced mouse models [14, 27]. While BALB/c mice did not show significant changes in TNF-α levels in response

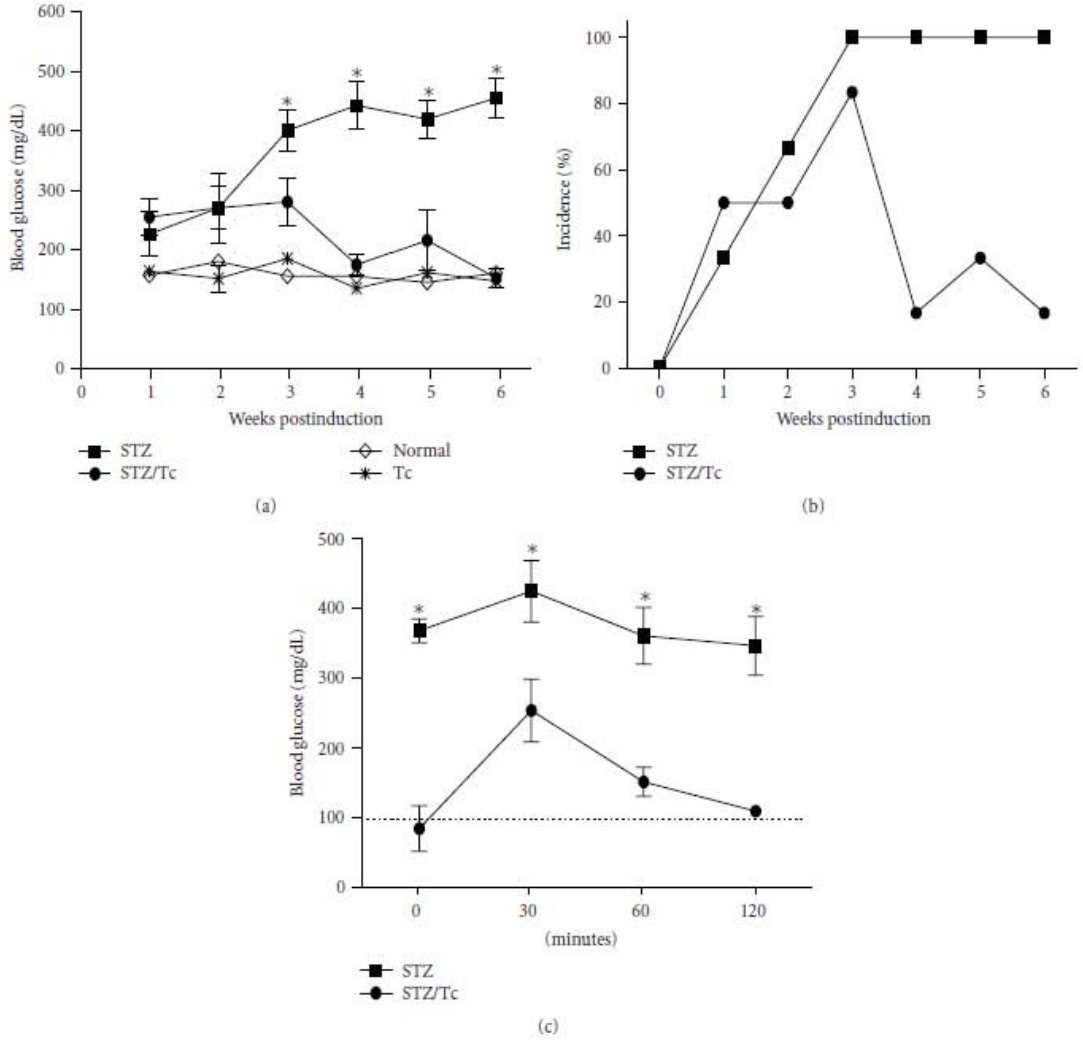

FIGURE 2: Hyperglycemia and diabetes incidence is modified by *T. crassiceps* infection in C57BL/6 mice. (a) Blood glucose levels throughout the MLDS protocol for uninfected C57BL/6 mice and mice infected with *T. crassiceps* for 6-weeks. (b) Percent incidence of diabetes between uninfected and *T. crassiceps*-infected C57BL/6 mice. (c) Intraperitoneal glucose tolerance test for MLDS-treated uninfected and *T. crassiceps*-infected mice. $^*P < .05$, $n = 8$.

to MLDS treatment (Figure 5(a)), uninfected C57BL/6J mice had high levels of TNF-α and IFN-γ in their sera (Figure 5(c), and data not shown). Interestingly, C57BL/6 mice that were previously infected with *T. crassiceps* and then treated with MLDS displayed lower sera TNF-α levels but maintained elevated levels of IFN-γ, as compared to uninfected mice (data not shown). We also evaluated the effect of *T. crassiceps* infection on the presence of the Th2-associated cytokine IL-4. At some experimental time points, IL-4 levels were significantly enhanced in both *T. crassiceps*-infected BALB/c and C57BL/6 mice that received MLDS whereas uninfected mice with T1D had lower levels of serum IL-4 (Figures 5(c) and 5(d)).

3.5. Presence of AAMϕ, but Not Tregs, in MLDS-Treated T. crassiceps-Infected Mice. In the last few years, new classes of regulatory cells that are induced by helminth infections have been identified. Tregs and AAMϕ have been implicated in the control of immune pathology associated with helminth infection, but both cell types have also been associated with suppression of the immune response [28, 29]. In order to determine whether these cells play a role in *T. crassiceps*-mediated diabetes prevention, we performed FACS analysis of peritoneal cells and T cells from the mesenteric lymph node. Following MLDS treatment, neither uninfected nor *T.*

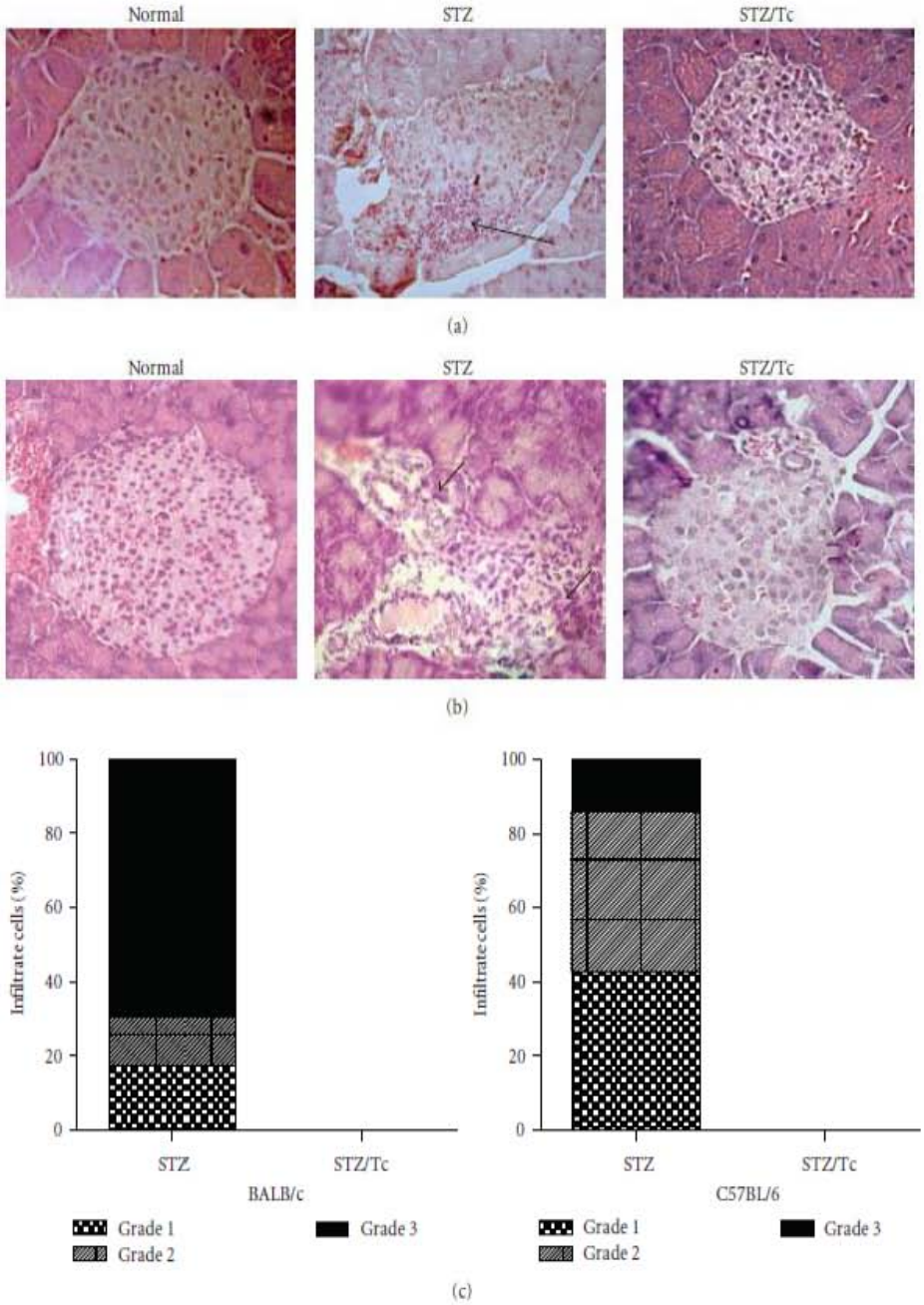

FIGURE 3: MLDS-mediated insulitis in BALB/c mice. All mice received MLDS for 5 consecutive days and were sacrificed 6 weeks later to harvest tissues for histopathology. (a) H&E stained BALB/c mouse pancreas sections: normal islet, STZ (uninfected and MLDS-treated), STZ/Tc (*T. crassiceps*-infected and MLDS-treated). (b) H&E stained C57BL/6 mouse pancreas sections. (c) Score of cell infiltrates in BALB/c and C57BL/6 islets. Note the lack of leukocyte infiltrates in *T. crassiceps*-infected mice. Arrows indicate infiltration. Magnification ×400.

crassiceps-infected mice displayed an increase in the population of Treg in the mesenteric lymph node (Figure 6(a)). Interestingly, *T. crassiceps*-infected mice displayed an increase in the percentage of AAMϕ in the peritoneum, as determined by the expression of the mannose receptor, CD23, PDL1, and MHCII (Figure 6(b)). To confirm the presence of AAMϕ, RT-PCR was performed to detect mRNA transcripts for Fizz-1 and PDL-1. These transcripts were present in peritoneal cells from *T. crassiceps*-infected mice treated with MLDS, but not in uninfected, MLDS-treated mice (Figure 6(c)).

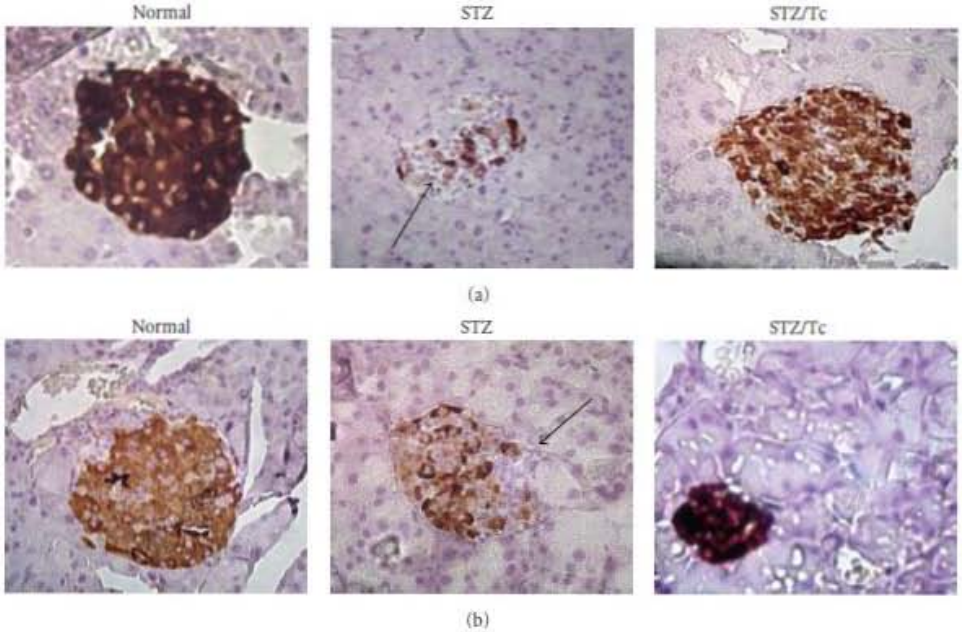

FIGURE 4: Insulin immunostaining of pancreatic islet cell from MLDS-treated mice. (a) Immunostaining of insulin in islets from BALB/c mice, insulin immunostaining in pancreatic islets of different uninfected (normal), uninfected and MLDS-treated mice (STZ), or *T. crassiceps*-infected MLDS-treated mice (STZ/Tc). Mice ($n = 4$) were sacrificed 6 weeks after the initial injection of MLDS and pancreata were processed for immunohistochemistry to specifically detect insulin. (b) Same as (a), just the sections belong to C57BL/6 mice. Note the lack of insulin staining in uninfected MLDS-treated mice. Magnification ×400.

4. Discussion

T. crassiceps infection and its associated antigens can induce Th2-type responses in vivo [20] and can modulate the immune response to bystander antigens or live infections [2]. *T. crassiceps*, like other helminth parasites, has the ability to manipulate and down-modulate the immune responses of its hosts [10, 18]. It is widely accepted that the initiation and development of T1D is mainly caused by an autoimmune cell-mediated destruction of β cells in the pancreas [15]. Genetic, immunological, and environmental factors can also influence the onset and development of T1D [30]. In this study, we found that *T. crassiceps* infection could alter the development of MLDS-induced diabetes in mice. This study demonstrates that *T. crassiceps*-infected mice develop a mild, and sometimes transient, form of T1D after MLDS treatment. In contrast, uninfected mice exhibited an accelerated, more severe form of T1D.

There are likely several factors that contribute to the protection by *T. crassiceps* infection against MLDS-induced diabetes. First, the prominent Th2 environment that is induced by *T. crassiceps* infection [19, 31] might counteract the proinflammatory responses that are necessary to generate complete MLDS-induced diabetes. Second, *T. crassiceps* infection might alter T cell recruitment to the pancreas. Third, *T. crassiceps* infection might induce a regulatory cell response that dampens inflammatory processes during MLDS-induced diabetes.

The findings of this study demonstrate that infection with *T. crassiceps* maintains high levels of IL-4 and that there is a slight reduction in serum TNF-α levels after infected mice are treated with MLDS. TNF-α has been implicated as a critical player in leukocyte-mediated islet damage [32]. Thus, this type of immune regulation might be responsible for the decrease in pathology and lower incidence of T1D observed in *T. crassiceps*-infected mice. Consistent with a putative protective role for Th2 type cytokines (IL-4), NOD mice that express IL-4 in their pancreatic β cells are protected from insulitis and autoimmune diabetes [33]. Other experimental models have demonstrated that helminth exposure (*Schistosoma mansoni*, *Trichinella spiralis*, *Hymenolepis diminuta* or *Heligmosomoides polygyrus*) to mice might drastically reduce the symptoms of autoimmune diseases such as Inflammatory Bowel Disease (IBD), Encephalomyelitis Autoimmune Experimental, and T1D, mainly by reducing IL-12 and IFN-γ production and enhancing IL-4 and IL-10 levels [34–45]. Female NOD mice that spontaneously develop diabetes due to Th1-mediated destruction of pancreatic β-cells are protected from developing diabetes when they are exposed to *S. mansoni* [34–36], and IL-4 has been shown to play an important role in this protection [46, 47]. It will be necessary to test our system in mice that either lack IL-4 or have been treated with a specific IL-4 inhibitor to fully understand the role that IL-4 plays in *T. crassiceps* protection against MLDS-induced diabetes.

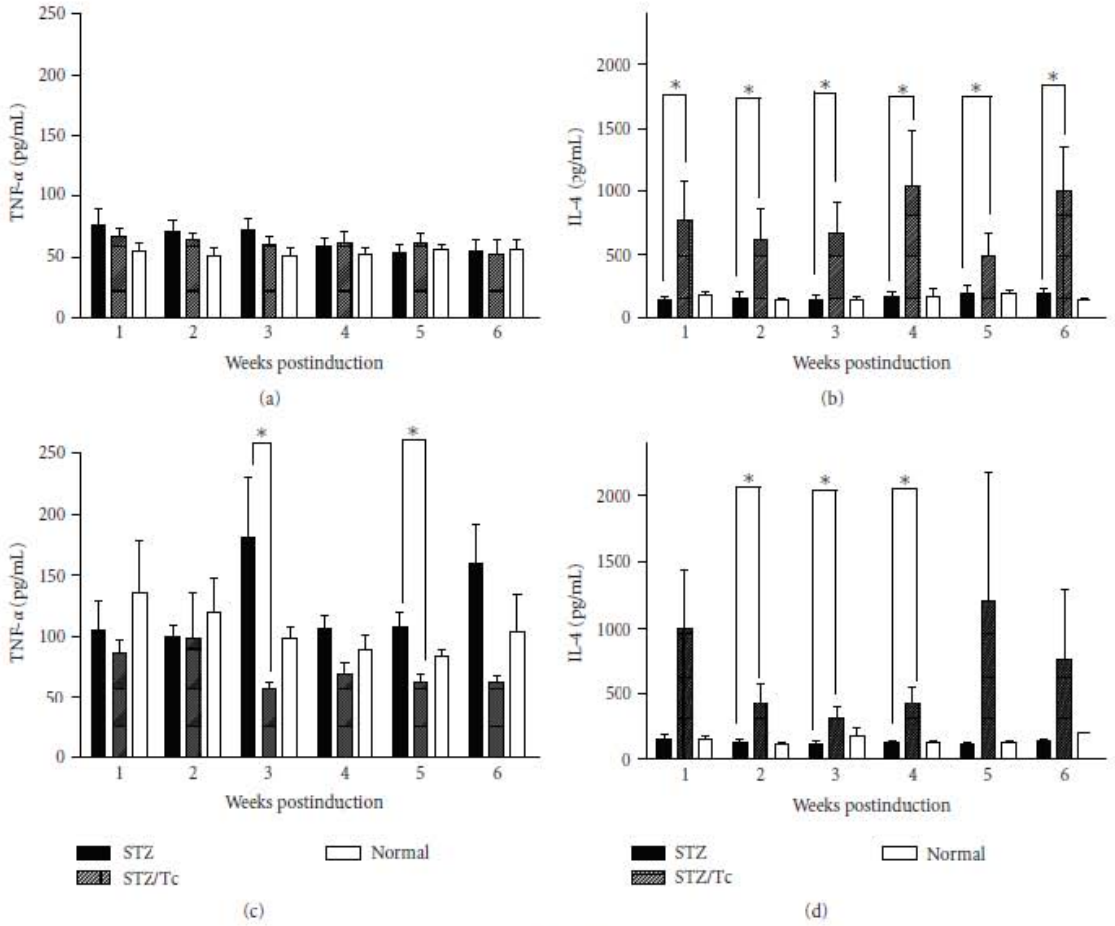

FIGURE 5: Cytokine profiles from the sera of mice treated with MLDS. BALB/c mice were bled at indicated time points and (a) TNF-α and (b) IL-4 were detected by ELISA. (c) TNF-α and (d) IL-4 for C57BL/6 mice. * $P < .05$, $n = 10$.

We found that *T. crassiceps* infection resulted in an increase in the population of F4/80$^+$ macrophages that express CD23, PDL1, MR, Arg1, Ym1, but not iNOS, an expression profile that is now used to identify AAMϕ. AAMϕ might function as bystander suppressors of the immune response. Alternatively, they might inhibit cell infiltration in the pancreas, given their established suppressive activity, which is mediated through cell-contact, where PD-L1 and PD-L2 play a preponderant role, or by releasing soluble factors [20]. In contrast, Treg cells were not detected in *T. crassiceps*-infected mice. Mice that were infected with *T. crassiceps* were protected from both hyperglycemia and lymphocyte infiltration into the pancreatic islet despite the absence of regulatory T cells. This is interesting given the recent findings that demonstrate the importance of Foxp3+ regulatory T cell expansion in mice infected with helminths [4–8]. Tregs induced by *S. mansoni* and its associated antigens have been implicated in diabetes prevention in NOD mice [36]. This discrepancy might be explained by differences in the classes of helminths used in these infection models, in the route of infection used, or by different suppressive mechanisms that are turned on by *T. crassiceps* versus *S. mansoni* [11, 19]. Only one published report demonstrates that macrophages are involved in preventing the pathology associated with IBD model [48]. Therefore, it is possible that AAMϕ might actively participate in dampening the pathology associated with MLDS-induced diabetes, perhaps by the known ability of AAMϕ to strongly suppress T cell responses.

Interestingly, MLDS-induced diabetes is associated with Th1-type responses [17] and C57BL/6 mice show a Th1-type response in the MLDS model [24]. We also show that *T. crassiceps*-infected C57BL/6 mice demonstrated a reversal of MLDS-induced diabetes even though they were more hyperglycemic at earlier time points following MLDS-treatment. Again, the specific regulatory cells and/or cytokines involved in these protective effects still need to be identified, however, according to our data, appears that AAMϕ it may have an

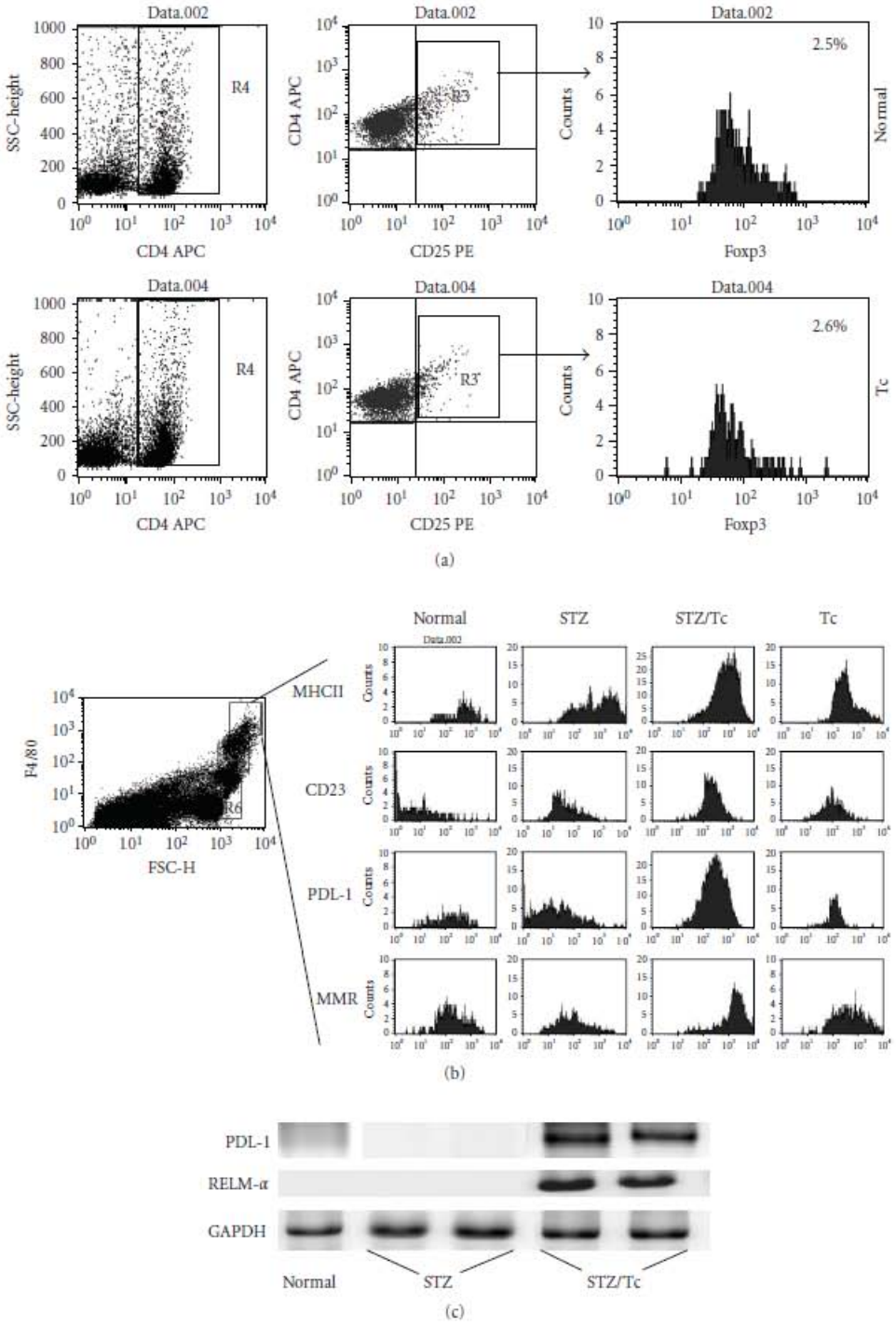

Figure 6: Flow cytometry analysis for the detection of regulatory T cells and alternatively activated macrophages. (a) Mesenteric lymph nodes from BALB/c mice were processed and cells were staining for Treg cell detection with anti-CD4, CD25, and Foxp3 (Tregs kit, Biolegend). (b) Peritoneal exudates cells stained with conjugated anti-F480, CD23, MR, PDL1, and MHCII and analyzed by flow cytometry. (c) RT-PCR analysis of PDL-1, RELM-α, and GAPDH in macrophages from uninfected and *T.crassiceps*-infected BALB/c mice.

important role instead T-regulatory cells that we could not detect in *T. crassiceps*-infected mice.

5. Conclusion

Our data support the notion that the protection from autoimmunity by helminth infection could be attributed to immunoregulatory mechanisms triggered by these parasites. Our study demonstrates for the first time that *T. crassiceps* infection protects against Multiple Low Dose Streptozotocin-Induced Diabetes, independently of the genetic background of the host. Furthermore, we present a possible protective role for AAMϕ since we did not detect enhanced Treg cells. The analysis of *Taenia*-released products and repeating our experiments under conditions of macrophage depletion will be necessary to fully understand the mechanisms involved in this observed protection.

Acknowledgments

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Review Article

Alternatively Activated Macrophages in Types 1 and 2 Diabetes

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Macrophages are innate immune cells derived from monocytes, which, in turn, arise from myeloid precursor cells in the bone marrow. Macrophages have many important roles in the innate and adaptive immune response, as well as in tissue homeostasis. Two major populations have been defined: The classically activated macrophages that respond to intracellular pathogens by secreting proinflammatory cytokines and reactive oxygen species and alternatively activated macrophages which are induced during Th2 responses displaying anti-inflammatory activities. Both macrophage populations are central players in diabetes, the first one triggering inflammatory responses which initiates insulitis and pancreatic β cell death during type 1 diabetes, whereas the second population decreases hyperglycemia, insulitis, and inflammation in the pancreas, thereby negatively regulate type 1 diabetes. Obesity is an important factor in the development of type 2 diabetes; classically activated macrophages are a dominant cell population involved in the establishment of the inflammatory profile, insulin resistance, and activation of inflammatory signals during the development and progression of this disease. In contrast, alternatively activated macrophages regulate the release of proinflammatory cytokines, attenuating adipose tissue inflammation. Here, we review the advantages and disadvantages of these two macrophage populations with regard to their roles in types 1 and 2 diabetes.

1. Macrophages

Mφs have important roles in the immune response and tissue homeostasis. The huge capacity of Mφs for phagocytosis renders them effective at microbial killing and the clearance of apoptotic and necrotic cells, and through their expression of MHC-II molecules and secretion of pro- and anti-inflammatory cytokines, they can also trigger CD4+ T-cell activation and differentiation into Th1, Th2, Th17, and Treg subsets [1–3]. Importantly, Mφs have diverse roles in the regulation of glucose and lipid metabolism, as well as in the inflammation of adipose tissue [4].

In recent years, it has been clearly demonstrated that macrophages display high plasticity depending on the microenvironment in which they are found. Two major macrophage phenotypes have been described, specifically, classically activated macrophages (CAMφs) and alternatively activated macrophages (AAMφs) [2]. CAMφs are induced by stimulation with Th1-cell-derived IFN-γ and microbial products, such as bacterial lipopolysaccharide (LPS) [5], and respond to microbial infection with an enhanced phagocytic microbicidal capability through the expression of the CAMs marker, inducible nitric oxide synthase (iNOS), which catalyzes the conversion of L-arginine into ROS, such as NO. These macrophages produce several proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), IL-1β, and IL-23, as well as toxic mediators, such as reactive oxygen species (ROS) and nitric oxide (NO), through the expression of inducible nitric oxide synthase (iNOS). These macrophages also have an enhanced antigen presenting ability [6].

In contrast, AAMφs are induced during Th2-type responses, such as those elicited by helminthic infection and during allergic responses. The activation of these macrophages is dependent upon stimulation with IL-4/IL-13 [16] through the IL-4Rα receptor [17] and signal transducer and activator of transcription factor 6 (STAT6) [18], as well as with several helminth antigens [19–22]. AAMφs produce

moderate levels of IL-10 and TGF-β and low or null levels of the proinflammatory cytokines secreted by CAMφs. Additionally, AAMφs produce urea, polyamines, and L-ornithine, due to the high expression of the enzyme arginase-1 (Arg-1), which competes for its common substrate, L-arginine, with iNOS, thereby lowering the levels of NO secretion [6, 23]. AAMφs have enhanced expression of Ym-1, which induces eosinophil recruitment [24]; these cells, in turn, can potentiate the Th2 response and the alternative activation of macrophages by the secretion of the anti-inflammatory cytokines IL-4/IL-13. Further, AAMφs can express high levels of PD-1 ligands (Program-Death 1), PDL-1 and PDL-2, thereby inhibiting the proliferative response of activated T-cells [25].

AAMφ populations have been identified as an essential part of the immune response against almost any helminth parasite, such as *Taenia crassiceps* [25, 26], *Brugia malayi* [27, 28], *Schistosoma mansoni* [29, 30], *Litomosoides sigmodontis* [31], *Nippostrongylus brasiliensis* [32], *Heligmosomoides polygyrus* [33], *Fasciola hepatica* [19], *Hymenolepis diminuta* [34], and *Echinococcus granulosus* [35].

Of importance for this paper, helminth-induced AAMφs have been linked with decreased T1D-triggering inflammation, as well as glucose tolerance induction during obesity [4], by which these macrophages may participate in inhibiting the initiation and development of both TD1 [7] and TD2 [15].

This paper focuses on the different roles that CAMφs and AAMφs display in both types of diabetes, emphasizing the role of AAMφs as essential players in diabetes regulation.

2. Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia as a result of the impairment of insulin secretion, its action, or both. The chronic hyperglycemia of diabetes is associated with dysfunction and failure of various organs, such as the eyes, kidneys, heart, and blood vessels [36]. It has been estimated that the number of deaths caused by diabetes worldwide is 4.6 million per year. Thus, diabetes remains a major cause of death and is considered to be an epidemic. Diabetes mellitus is divided into two categories: type 1 diabetes (T1D) and type 2 diabetes (T2D), and at least 90% of all cases belong to the latter [37].

2.1. Type 1 Diabetes. T1D is an autoimmune disease that has increased in prevalence over the last 30 years in developed countries. It is known that more than 5.3 million people in the world have T1D, and more than 218,000 may develop the disease each year [40]. T1D is caused by the selective destruction of the insulin-producing β cells located in pancreatic Langerhans' islets by autoantigen-specific inflammatory T cells. Insulin, glutamic acid decarboxylase (GADA/GAA), and protein tyrosine phosphatase (IA-2AA) are the most common autoantigens involved in this process. When the majority of β cells are destroyed, the pancreas's ability to secrete insulin in response to blood glucose levels is impaired, resulting in the disruption of glucose homeostasis [36].

CAMφs and CD4+ and CD8+ autoreactive lymphocytes are the first cells that infiltrate the Langerhans islets, and the levels of cytokines, such as TNF-α, IL-1β, and IL-6, as well as NO, are increased in the pancreas during inflammation (Figure 1), where they activate different signaling pathways [38]. IL-1β and TNF-α induce the NF-κB (nuclear factor κB) signaling pathway, which promotes apoptosis of β cells by increasing the expression of FAS. TNF-α and IFN-γ act synergistically to activate the transcription factor signal transducer and activator of transcription-1 (STAT-1) signaling, thus inducing iNOS overexpression and secretion of NO and thereby promoting apoptosis of β cells by the p53 pathway [38, 39]. Free radicals, in turn, can induce apoptosis and necrosis of β cells by activating the caspase pathway and inducing excessive cell stress, respectively [39]. During this process, chemokines, such as MCP-1 (or CCL2), are also secreted; this chemokine is important in the recruitment of CAMφs, inflammatory monocytes, dendritic cells, and T cells into the pancreatic islets [40, 41]. Another cytokine that has been involved in T1D is the macrophage migration inhibitory factor (MIF). MIF is associated with MCP-1, which facilitates monocyte transmigration [42]. In a mouse model with MLD-STZ, the levels of MIF were elevated in diabetic mice, and the use of MIF inhibitors reduced the inflammatory response and insulitis [43].

A study performed in diabetic patients showed increased numbers of monocytes, as well as higher levels of IL-1β, IL-6, and TNF-α, in the pancreas of sick patients compared with healthy people. The enhanced expression of CD80 and PDL1 in the infiltrating monocytes suggests a proinflammatory profile for these cells [44]. Several studies have attempted to verify the role of CAMφs as important cells in the initiation and development of T1D. In experimental models, Martin et al. [41] demonstrated that the increased expression of CCL2 (using RIPCCL2 transgenic mice) promotes the recruitment of inflammatory monocytes to the pancreatic islets, thereby initiating inflammation and destruction of β cells. These data suggest that monocytes are needed for the development of diabetes. Also, the experimental depletion of CAMφs in NOD mice by the intraperitoneal injection of clodronate liposomes resulted in a decrease in insulitis and inflammation [45, 46].

Recently, a new subpopulation of CD4+ T lymphocytes, known as Th17 cells, have been described, which are characterized by their ability to secrete high levels of IL-17, thereby promoting an inflammatory profile. The differentiation of Th17 cells is dependent upon IL-6 and transforming-growth factor-β (TGF-β) stimulation, and the presence of this subpopulation of CD4+ cells has been correlated with the onset and progression of autoimmune diseases, such as T1D [47]. IL-23 is an inflammatory cytokine involved in the expansion and commitment of Th17 cell populations, and one of its main sources is CAMφs. In diabetic mice induced by streptozotocin (STZ), it has been shown that the administration of IL-23 increases IL-17, TNF-α, and IFN-γ secretion, which is associated with the onset of extremely severe T1D, implicating CAMφs in the recruitment, differentiation, and expansion of pathogenic Th17 lymphocytes, contributing to β cell death and T1D induction [48]. Therefore, CAMφs

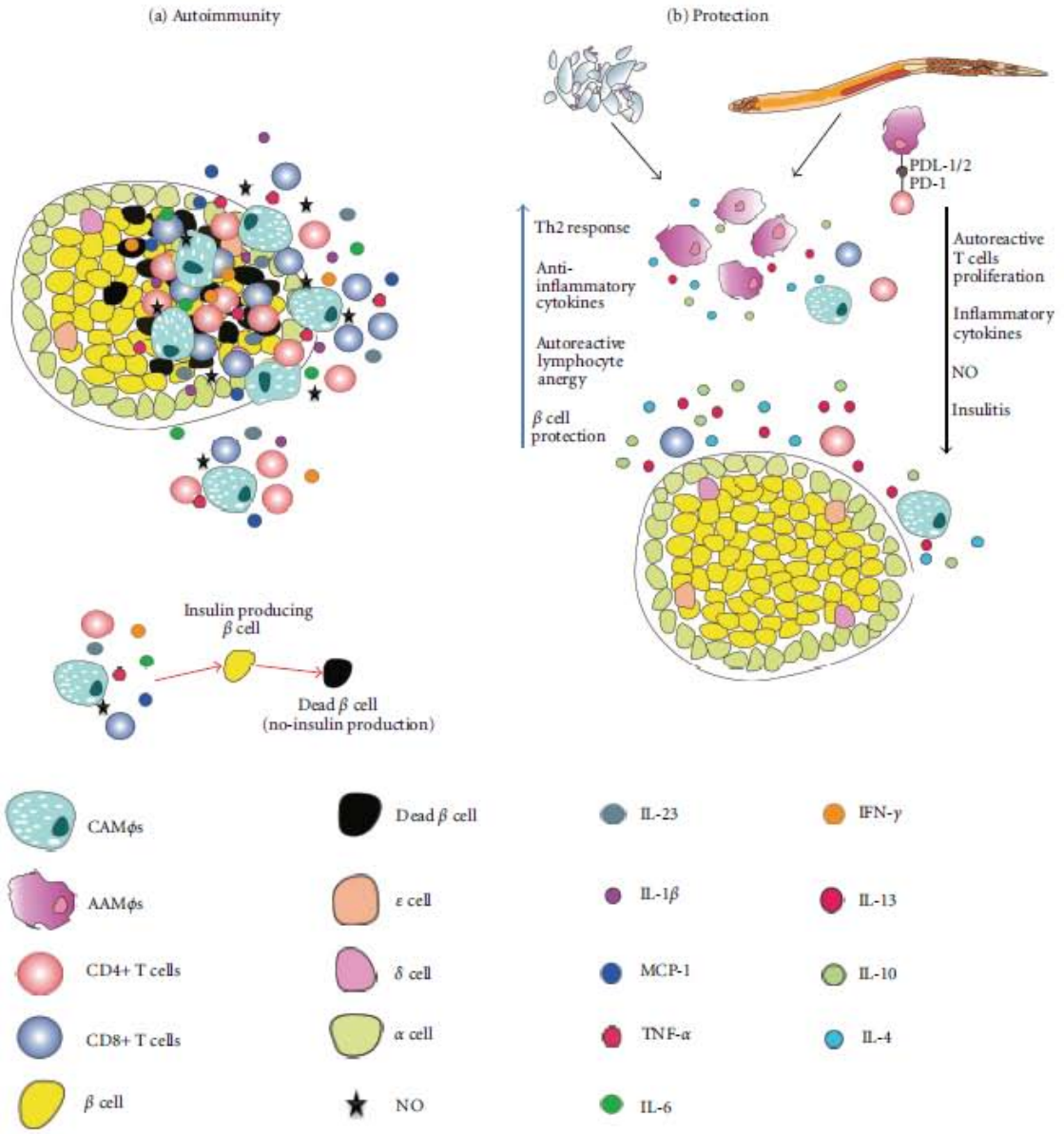

FIGURE 1: (a) In type 1 diabetes, CAMφs and autoreactive T cells are the first cells that infiltrate the islets of Langerhans and release proinflammatory cytokines and NO, which induce β cell apoptosis or necrosis; (b) the release of anti-inflammatory cytokines, AAMφ induction and PD-1/PD-ligand-dependent lymphocyte anergy induction by helminths, the antigens of which have the ability to decrease NO, as well as proinflammatory cytokine, secretion, thereby reducing insulitis and β cell death.

and Th17 cells, together with CD8+ cytotoxic T cells, are considered to be the main cell populations favoring the development of T1D.

However, certain pathogens (mainly viruses) can induce the development of T1D, including Rubella, enterovirus, rotavirus, cytomegalovirus, and mumps, by diverse mechanisms [49]. Several viruses may break self-tolerance by the expression of viral antigens; additionally, certain viral proteins show homology with autoantigens of β cells (known as molecular mimicry). Furthermore, several viruses can express superantigens, which results in an increase in the autoreactive T-cell populations, or induce the cytolysis of β cells, including Coxsackievirus [50] and Encephalomyocarditis (EMC) virus [51]. In the case of humans, rubella virus

Table 1: Helminths that reduce types 1 and 2 diabetes.

Helminths	Disease/model	Infection/antigen	Effect	Reference
Taenia crassiceps	T1D/MLD-STZ	Inf	Increased Th2 response, AAMφ induction, and decreased TNF-α, therefore less hyperglycemia and no insulitis	[7]
Schistosoma mansoni	T1D/NOD/MLD-STZ	Inf/Ag	Increased anti-inflammatory cytokines, such as IL-4, IL-10, IL-5, and IL-13, as a result, loop of Th2 response; Treg, eosinophil, and AAMφ generation	[8–11]
Heligmosomoides polygyrus	T1D/NOD	Inf	Th2 response induction; IL-4, IL-13, and IL-10 augmentation; AAMφs in pancreatic and peripheral lymph nodes; inflammation and insulitis reduced; no Treg generation	[12]
Litomosoides sigmodontis	T1D/NOD	Inf/Ag	High IL-4 and IL-5; AAMφ and Treg induction; reduced inflammation and glycemia	[13]
Trichinella spiralis	T1D/NOD	Inf/Ag	Amplification of Th2 response; less injury in pancreas and glycemia	[14]
Nippostrongylus brasiliensis	T2D/obese	Inf	Th2 response; recruitment of eosinophils and AAMφs; decreased obesity and insulin resistance	[15]

infection correlates with an increased incidence of T1D, and one possible mechanism of induction is molecular mimicry. Other examples are rotavirus and reovirus, which have been shown to induce lysis of β cells and release of autoantigens, suggesting the first mechanism of induction of T1D [49, 51]. Conversely, other pathogens may have protective roles and T1D. Epidemiological observations have pointed out an increase in the incidence and prevalence of T1D and other autoimmune diseases, mainly in developed countries, which have been correlated with a decrease in the incidence of bacterial and parasitic infections, particularly helminth infections. These observations prompted the proposal of the *hygiene hypothesis*, which states that the lack of intense infections that actively modulate the balance of the immune response toward Th2 or anti-inflammatory profiles (such as those that can be found in helminth infections) favors the induction of strong Th1 immune responses against autoantigens, thereby favoring the development of autoimmune responses [40, 52].

Helminths share a unique ability to exert profound regulatory effects on the immune system of their hosts by inducing strong Th2-type responses and increasing the numbers of regulatory cell populations, such as Tregs and AAMφs. The results of several experiments in murine models of autoimmunity and its regulation by helminth infections support the protective role of helminth-induced Th2 responses proposed by the hygiene hypothesis [3, 53, 54]. For example, it has been shown that the infection of nonobese diabetic (NOD) mice with *Heligmosomoides polygyrus* has a protective effect in T1D, resulting in the regulation of hyperglycemia and reduced incidence of diabetes; these effects were accompanied by reduced numbers of macrophages, dendritic cells, and CD4+ and CD8+ T cells in the inflammatory infiltrate in the pancreas, as well as a reduction on β cell damage. Importantly, higher numbers of AAMφs were found in the pancreatic and peripheral lymph nodes of NOD mice compared to noninfected mice [12]. Interestingly, in other studies, the experimental infection of mice with *Schistosoma mansoni* or their treatment with either helminth or soluble worm extracts (SWA) or soluble egg antigen (SEA) could prevent diabetes in NOD mice, with a direct relationship being observed between the lower incidence of T1D and reduced insulitis and higher numbers of AAMφs [8–10]. Other regulatory cell populations, such as Treg cells, which can inhibit inflammation and suppress several autoimmune diseases, including T1D, also increased in number during *Schistosoma mansoni* infection and antigen administration [10]. Other parasites, such as *Litomosoides sigmodontis*, have also been shown to reduce T1D [13]. We have shown that previous *Taenia crassiceps* infection of diabetic mice, which were induced by multiple low doses of streptozotocin (MLD-STZ), significantly decreased the incidence of T1D, hyperglycemia, and the inflammatory infiltration of islets of Langerhans. These effects were accompanied by a significant increase in the secretion of IL-4 and the expansion of the AAMφs population compared with noninfected, diabetic mice, suggesting that AAMφs induced by *T. crassiceps* infection can be important in the protection against T1D [7]. In a recent study, the adoptive transfer of AAMφs, which were induced *in vitro* by IL-4 and IL-13, into diabetic mice reduced kidney injury, hyperglycemia, and insulitis in the pancreas, clearly suggesting that AAMφs may have a protective role against T1D [55]. In another recent study, the adoptive transfer of AAMφs, which expressed PDL-2, FcγRIIb, IL-10, and TGF-β prevented 80% of NOD mice from developing this disease [56]. Collectively, these data suggest that AAMφs may have important roles in the inhibition and prevention of T1D (Table 1 and Figure 1).

2.2. Type 2 Diabetes. T2D is a metabolic disease, and its incidence has increased significantly in recent years. It is estimated that in 2000, there were approximately 171 million

people with this disease, and it has been predicted that by 2030, the prevalence of T2D will increase to 366 million people [57]. T2D is characterized by a peripheral resistance to the action of insulin and a rise in insulin production by β cells in a process called "compensatory hyperinsulinemia" to force glucose uptake in peripheral tissues. Regardless, during T2D, there is a chronic deficiency of glucose uptake and insulin action, mainly in the liver, skeletal muscle, and adipose tissue (AT), causing hyperglycemia, hypercholesterolemia, and hyperlipidemia [58, 59].

AT is composed of adipocytes, preadipocytes (which are immature adipocytes that have not yet loaded any lipids), endothelial cells, leukocytes, fibroblasts, and macrophages [60]. During obesity, lipid accumulation causes a high degree of stress on adipocytes, activating them and promoting the production and subsequent release of free fatty acids (FFA), proinflammatory adipocytokines (such as leptin and resistin), and cytokines, such as IL-1β, IL-6, TNF-α, MCP-1, and MIF, as well as ROS [61–63], ensuring that in addition to its well-known capacity to store energy, AT has the capability to function as an endocrine organ. In fact, this endocrine ability of AT triggers inflammation, leading to insulin resistance and the development of T2D.

Several data show that macrophages are recruited into AT and classically activated due to adipocytokine secretion, contributing to the establishment of an inflammatory profile and insulin resistance in this tissue. A deficiency of MCP-1 (CCL2) or CCR2 (CCL2 receptor) in mice during obesity results in the impairment of CAMφ recruitment to adipose tissue, thus impeding the induction of insulin resistance by a high-fat diet (HFD) [64, 65] and suggesting an important role for CAMφs in T2D initiation and development (see Figure 2). Additionally, the stressed AT secreted the adipocytokines leptin and resistin, which have been implicated in the recruitment and activation of monocytes and CAMφs in adipose tissue, inducing these cells to produce higher levels of TNF-α, IL-12, and IL-6 [61]. Besides the production of resistin by stressed AT, stressed AT also induces the expression of MCP-1 and cellular adhesion molecules, such as V-CAM and ICAM, in adipose tissue and its vascularization [66]. Furthermore, FFA can be recognized by Toll-like receptors (TLRs) with low affinity, leading to the activation of macrophages, which release more TNF-α [67, 68]. TNF-α (one of the cytokines most abundantly secreted by CAMφs) has the ability to reduce the expression of important genes in the glucose regulation process, such as the glucose transporter GLUT-4 [4]; in fact, TNF-α receptor knock out mice are resistant to diabetes induction [69], suggesting that the endocrine function of AT is important in the recruitment and activation of CAMφs and the induction of insulin resistance. Consistent with these observations, a recent report on a model of T2D (induced with a single high dose of streptozotocin) in MIF KO mice showed that these mice had a reduced inflammatory response, such as reduced TNF-α production, and failed to develop T2D, demonstrating that MIF is also important in promoting the disease [70].

Secretion of IL-1β, TNF-α, and ROS by AT CAMφs induces the activation of JNK and NF-κB signaling in various leukocytes. NF-κB is a transcription factor with an important role in the induction of inflammatory responses and the activation of CAMφs, whereas JNK (c-Jun amino-terminal kinase), also known as the protein kinase activated by stress (SAPK), is activated by oxidative stress. Therefore, the activation of these signaling pathways induces the production of more IL-1β, TNF-α, and MCP-1 and high levels of iNOS expression, contributing to insulin resistance in different tissues [71–73].

When insulin binds to its receptor, IRS-1 and IRS-2 (insulin-receptor substrates 1 and 2) are recruited to its cytoplasmic region, which permits the binding and activation of two important kinases, the first of which is PI3K (phosphatidylinositol 3-kinase), and the second of which is AKT (a protein kinase B) [74]. Once activated, these kinases can regulate glucose and lipid metabolism. However, activated JNK can induce the phosphorylation of serine residues on IRS-1/2, inhibiting their ability to couple to PI3K and thereby promoting insulin resistance. In fact, the expression of JNK and NF-κB is increased in diabetic patients [73], suggesting an important role for these molecules in diabetes. In myeloid-specific I$\kappa\kappa$-β (an activator of NF-κB)-deficient mice, a decrease in proinflammatory cytokine production (IL-1β, IL-6, TNF-α, and MCP-1) and the inhibition of NF-κB activation has been reported, avoiding, in this way, the development of insulin resistance [75].

CAMφs have been confirmed to be directly involved in diabetes because it has been found that 30% of the transcripts expressed in the adipose tissue of HFD-treated mice encode characteristic macrophage proteins associated with this subpopulation [76]. Also, the expression of transcripts for MIP-1α, MCP-1, MAC-1, F4/80, and CD68 was associated with insulin subproduction and TNF-α release [77]. In addition, macrophage polarization to CAMφs had a direct relationship with the development of lipid droplets [78]. These characteristics relate the activation of CAMφs to the promotion of AT accumulation and insulin resistance.

Interestingly, a macrophage phenotypic switch has been reported in the AT of HFD-treated mice compared with normal diet-treated mice. Lumeng et al., 2007 [4], reported the presence of a natural AAMφ population within the AT of lean mice, and interestingly, the phenotype of these cells shift to CAMφs when the mice were HFD-treated. The authors also showed that the IL-10 produced by AAMφs had the ability to block the pathological effects of TNF-α in adipose tissue during insulin sensitivity [4, 78, 79], suggesting that while CAMφs have insulin resistance-inducing effects, AAMφs have a protector role within AT. Recently, another inflammatory chemokine has been shown to be involved in the resistance to insulin and T2D. A-ZIP transgenic mice (these animals are insulin-resistant and hyperlipidemic), which have a deficiency in MCP-1, displayed decreased hyperglycemia, hyperinsulinemia, and hepatomegaly; moreover, these mice had increased levels of markers for AAMφs, such as *Arg1* and *Chi313* [80].

Also of note, PPARs are ligand-dependent transcription factors that have important functions in FA transport, synthesis, storage, mobilization, activation, and oxidation. Three distinct types of PPARs have been characterized: PPARα, PPARδ, and PPARγ. PPARα and PPARδ are involved in the

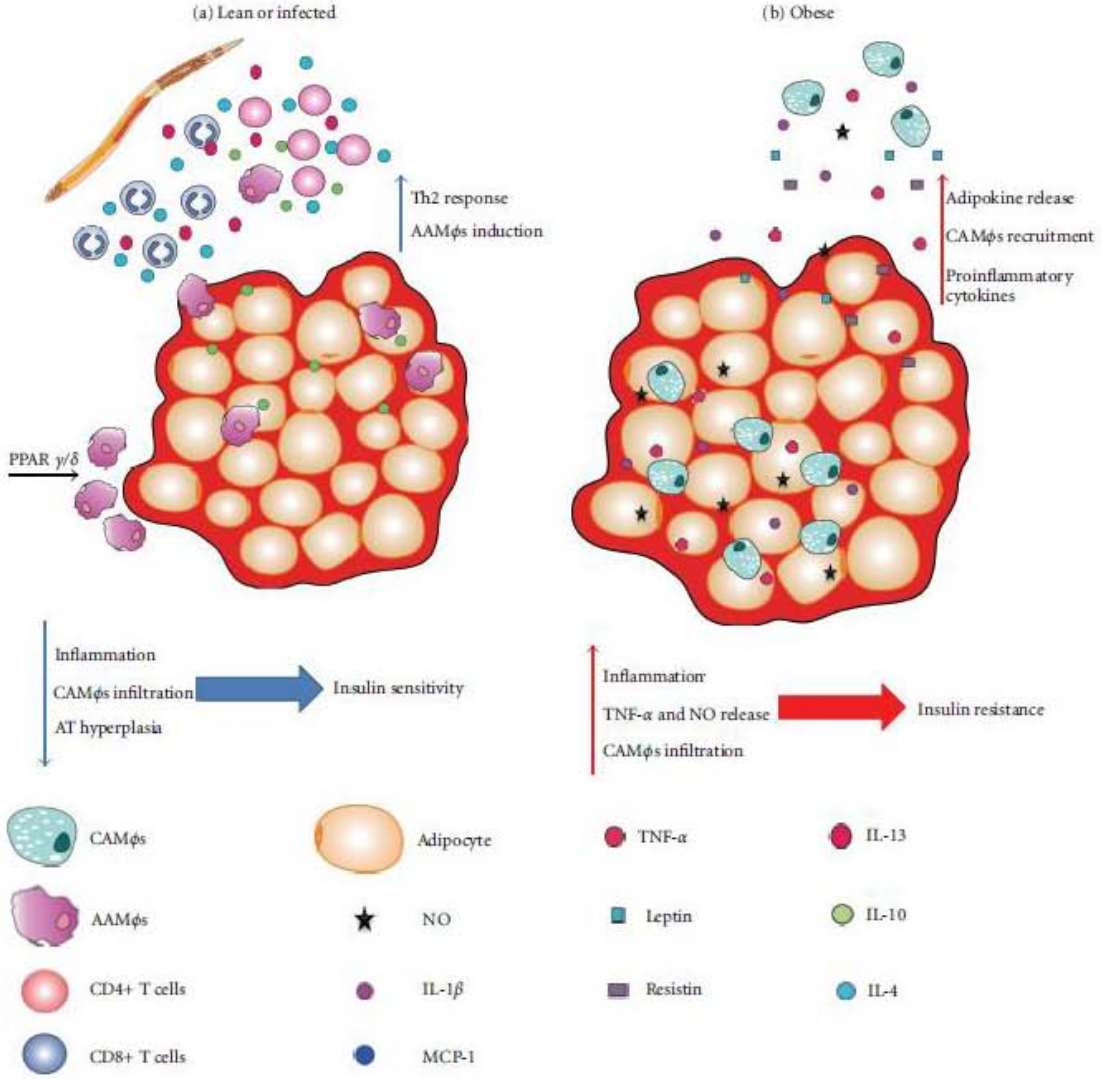

FIGURE 2: (a) Lean individuals have AAMφs in their AT, which protect them from insulin resistance by secreting IL-10. An helminth infection can recruit Th2 lymphocytes, IL-4/13-secreting eosinophils and AAMφs, thereby increasing protection. The natural AAMφ population in the lean AT is sustained by PPARγ/δ; (b) obesity induces resistin and leptin secretion, as well as proinflammatory adipocytokines, thereby promoting CAMφ recruitment into the AT. CAMφs in turn induce insulin resistance by secreting NO and TNF-α.

oxidation of FFA, while PPARγ contributes to adipogenesis and the storage of FA. PPARγ expression is induced in Mφs by IL-4/IL-13 [81–83]. Recent reports have shown that PPARγ is required for AAMφs induction and maturation, and the absence of this molecule enhances obesity and insulin resistance in HFD mice [81]. Moreover, PPARδ-deficient Kupffer cells cannot be alternatively activated, predisposing mice to develop hepatic steatosis and insulin resistance [84]. As mentioned above, AAMφ development is dependent on IL-4/IL-13 stimulation, which activates the transcription factor STAT-6. STAT-6-deficient mice are more prone to obesity, and oxidative stress in their AT makes them more susceptible to T2D development, which, in turn, is associated with the absence of AAMφs [85].

The role of other cells in the regulation of insulin sensitivity is recognized principally because of evidence in experimental models. Eosinophil-deficient mice have a smaller AT-AAMφ population and gain more weight, which indicates that eosinophils are an important source of IL-4 in adipose tissue [15]. Likewise, *Nippostrongylus brasiliensis* infection

induced the recruitment of eosinophils and AAMφs, which promoted a strong Th2 response and decreased obesity and insulin resistance [15], suggesting that eosinophils contribute to AAMφ induction and prevent T2D.

Collectively, these findings suggest that adipose tissue is an important source of inflammatory molecules during obesity and can induce insulin resistance due to the increased recruitment of CAMφs, which, in turn, can amplify the inflammatory response, promoting development of T2D, while high numbers of AAMφs in the adipose tissue have been involved in glucose tolerance and diabetes prevention (Figure 2).

3. Conclusions

There is no doubt that the incidence of diabetes has increased in recent years, perhaps reflecting changes in lifestyle with regard to diet and/or hygiene. One explanation for the increased incidence of T1D is the hygiene hypothesis, which suggests that low or null exposure to parasites, especially helminths or their antigens, promotes the development of autoreactive leukocytes that attack β cells, initiating the disease. Helminth infections in mice with T1D have proved to prevent the inflammatory cascade through a mechanism associated with AAMφ induction. AAMφs have been implicated in the regulation of other autoimmune diseases, such as experimental autoimmune encephalomyelitis [86] and autoimmune colitis, suggesting that AAMφs have a strong immunoregulatory role in the induction of autoantigen tolerance [87]. Therefore, it is likely that these cells are the main players in the regulation of T1D.

The importance of AAMφs extends beyond the regulation of autoimmunity, which we reviewed in this paper. AAMφs can also inhibit the development of T2D, mainly by reducing obesity and insulin resistance, two major etiological factors in the induction of this disease, while CAMφs are associated with increasing inflammation, obesity, and insulin resistance. Interestingly, the use of helminth parasites to induce AAMφs has proved to be effective in disease treatment by reducing hyperglycemia, obesity, and the incidence of T2D.

Finally, while CAMφs have a major role in the injury and inflammatory response in diabetes, AAMφs appear to reduce inflammation during type 1 and type 2 diabetes, suggesting that these macrophage populations may be therapeutic targets. Thus, based on the results of the various reports reviewed in this paper, we can highlight the possible therapeutic use of diverse immune-modulatory molecules to counteract or negatively influence specific inflammatory and cytotoxic T-cell-activating properties of macrophages.

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