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FES-IZTACALA

"**Impacto de la actividad anti-inflamatoria de *Taenia crassiceps* en el desarrollo del cáncer de colon asociado a colitis**"

TESIS

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Índice

Abreviaturas

AOM	Azoximetano
AAMs	Macrófagos activados alternativamente
AKT/PKB	Proteínas quinasa de serina/treonina o proteína quinasa B
APC	Adenomatous polyposis coli
CCR	Cáncer colorrectal
CAC	Cáncer de colon asociado a colitis
CD	Enfermedad de Crohn
CU	Colitis Ulcerativa
DC	Células dendríticas
DNMT1	DNA metiltransferasa 1
DSS	Dextran Sulfato de Sodio
DT1	Diabetes tipo 1
FAP	Adenomatosis poliposa familiar
HNPCC	Cáncer colorrectal hereditario no asociado a poliposis
IBD	Enfermedades inflamatorias intestinales
ICAM-1	Moléculas de adhesión intercelular 1
IEC	Células epiteliales del colon
LPS	Lipopolisacaridos
MMR	Genes mismatch repair
NF-κB	Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas

PIAS3	Inhibidor de proteína de STAT3 activado
PI3K	Fosfatidilinositol-3-quinasas
PTEN	Fosfatidilinositol-3,4,5-trisfosfato 3-fosfatasa
RNI	Intermediarios de nitrógenos reactivos
ROS	Especies de oxígeno reactivas
SH-2	Dominio de homología 2 Src
SHP2	Proteína tirosina-fosfatasa 2
SOCS3	Supresor de señalización de citocinas
STAT	Transductor de señal y activador de transcripción
TcES	Productos excretados/secretados de *Taenia crassiceps*
Th1/2	Célula T cooperadora 1/2
TLR	Receptor tipo Toll
TNF-α	Factor de necrosis tumoral alfa
TyK2	Tirosina quinasa 2

Abstract

Inflammation is currently considered a hallmark of cancer and plays a decisive role in different stages of tumorigenesis, including initiation, promotion, cancer progression, metastasis and resistance to antitumor therapies. Colitis-associated colon cancer (CAC) is one of the most common cancers and is closely related to chronic or dysregulated inflammation. In addition to inflammation, other extrinsic factors such as infections with pathogens such as viruses, bacteria and helminth parasites can alter the progression of cancer, for example, several reports confirm the ability of some parasitic infections to modulate the development of cancer, in many cases favoring tumor progression but in others inhibiting tumorigenesis.

The ability of helminthic infections to modulate inflammatory responses in some diseases is widely documented, for example, we previously reported that infection by *Taenia crassiceps* regulates experimental acute colitis by inducing alternatively activated macrophages (AAMs) and inhibiting the production of inflammatory cytokines. However, its immunomodulatory function during the development of cancer remains completely unknown. In this thesis we have analyzed the role of the anti-inflammatory response induced by *Taenia crassiceps* in the course of CAC development, where we showed that the extraintestinal infection by *T. crassiceps* in CAC inhibited the inflammatory responses in colon as well as the formation of tumors and prevented the loss of goblet cells. There was also an increase in the expression of IL-4 and markers associated with AAMs in the colon tissue and negative immunomodulation of the expression of proinflammatory cytokines. In addition, *T. crassiceps* infection prevented the positive regulation of β-catenin and the expression of CXCR2 observed in CAC mice, we also observed reduction in the number of monocytes $CD11b^{+}Ly6C^{hi}CCR2^{+}$ in circulation and in colon tissue. On the other hand, previous reports suggest an immunoregulatory effect of the products derived from *T. crassiceps* very similar to those observed with a full infection with *T. crassiceps.* The aim of the following investigation was to determine the effect of the excreted / secreted products of *Taenia crassiceps* (TcES) as a treatment in the CAC model. Here we found that after induction of CAC, treatment with

TcES reduced inflammatory cytokines such as IL-1β, TNF-α, IL-33, IL-17 and significantly attenuated colon tumorigenesis. This remarkable effect was associated with the inhibition of STAT3 and NF-κB phosphorylation. In addition, in 3D cultures, TcES promoted the reorganization of the actin cytoskeleton in RKO cell line, altering the cell morphology and forming the colonospheres, characteristics associated with a low degree of aggressiveness. In conclusion, the previous infection by *Taenia* and its TcES products inhibit the development of CAC through the negative regulation of proinflammatory and protumorigenic signaling pathways.

Resumen

La inflamación actualmente se considera una característica del cáncer y desempeña un papel decisivo en diferentes etapas de la tumorigénesis, que incluyen iniciación, promoción, progresión del cáncer, metástasis y resistencia a terapias antitumorales. El cáncer colorrectal asociado a colitis (CAC) es uno de los cánceres más comunes y está estrechamente relacionado con la inflamación crónica o desregulada. Además de la inflamación, otros factores extrínsecos como las infecciones con agentes patógenos como virus, bacterias y parásitos helmintos pueden alterar la progresión del cáncer, por ejemplo, diversos reportes confirman la capacidad de algunas infecciones parasitarias de modular el desarrollo del cáncer, en muchos casos favoreciendo la progresión tumoral y en otros inhibiendo la tumorigénesis.

Es ampliamente documentada la capacidad de las infecciones helmínticas de modular las respuestas inflamatorias en algunas enfermedades. En nuestro laboratorio hemos reportamos que la infección por *Taenia crassiceps* regula la colitis aguda experimental induciendo macrófagos activados alternativamente (AAMs) e inhibiendo la producción de citocinas inflamatorias, sin embargo, su función inmunomoduladora durante el desarrollo del cáncer sigue siendo completamente desconocida. En esta tesis analizamos el papel de la respuesta antiinflamatoria inducida por *Taenia crassiceps* en el curso del cáncer de colon asociado a colitis (CAC). Las evidencias obtenidas indican que la previa infección extraintestinal por *T. crassiceps* en CAC inhibió las respuestas inflamatorias en colon, la formación de tumores e impidió la pérdida de células caliciformes. Estos efectos se suman a un aumento de la expresión de IL-4 y marcadores asociados a AAMs en el tejido de colon y una inmunomodulación negativa de la expresión de citocinas proinflamatorias. La infección por *T. crassiceps* impidió la regulación positiva de la β-catenina y la expresión de CXCR2 observada en los ratones CAC. Así como la reducción en el número de monocitos $CD11b^{+}Ly6C^{hi}CCR2^{+}$ en circulación y en tejido de colon. Por otro lado, reportes previos sugieren un efecto inmunoregulador de los productos derivados de *T. crassiceps* muy semejantes a los observados por la infección por *Taenia*.

En este trabajo de tesis se evaluó además el efecto de los productos excretados/secretados de *Taenia crassiceps* (TcES) como tratamiento en el modelo de CAC. El tratamiento con TcES en ratones con CAC, redujo las citocinas inflamatorias como IL-1β, TNF-α, IL-33, IL-17 y atenuó significativamente la tumorigénesis en colon. Estos efectos se acompañaron con la inhibición de la fosforilación de STAT3 y NF-κB. Adicionalmente identificamos que los TcES interfirieren con la proliferación de una línea de células epiteliales humanas transformada de colon (RKO). Además, en cultivos 3D, TcES promovió la reorganización del citoesqueleto de actina, alterando la morfología celular y formando las colonosferas, características asociadas con un bajo grado de agresividad. En resumen, concluimos que la infección previa con *T. crassiceps* así como los TcES inhiben el desarrollo de CAC mediante la regulación negativa de vías de señalización proinflamatorias y protumorigénicas.

GENERALIDADES

Cáncer de colon asociado a colitis (CAC)

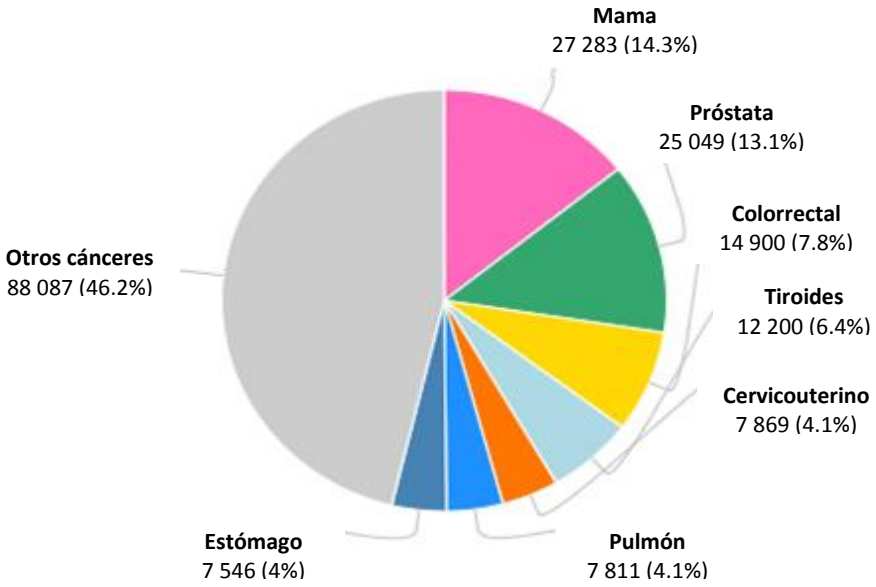
El cáncer en el sentido más amplio se refiere a más de 277 tipos diferentes de enfermedades las cuales poseen varios estadios, lo que indica que múltiples mutaciones genéticas y factores ambientales están involucrados en su patogénesis. Durante la carcinogénesis, las células tumorales adquieren capacidades biológicas conocidas como "hallmark" que incluyen; proliferación sostenida, evasión de supresores de crecimiento, resistencia a la muerte celular, inmortalidad replicativa, inducción de angiogénesis, invasividad y metástasis, inestabilidad genómica e inflamación, esta última fomenta múltiples funciones de los "hallmark". Actualmente han sido agregados dos hallmark a esta lista: la reprogramación del metabolismo energético y la evasión de la destrucción inmune [1].

La incidencia de cáncer y la mortalidad por ésta enfermedad están creciendo rápidamente en todo el mundo. Las razones son complejas, el envejecimiento, como el crecimiento de la población participan en los cambios en la prevalencia [2].

El cáncer colorrectal (CCR) es el tercer tipo de cáncer más frecuente y la segunda causa de muerte por cáncer en todo el mundo, además es el segundo cáncer más común en mujeres y el tercero en hombres [3]. Alrededor del 20% de los casos de CCR son asociados a factores genéticos, tal es el caso de adenomatosis poliposa familiar (FAP) y el cáncer colorrectal hereditario no asociado a poliposis o síndrome de Lynch (HNPCC), los cuales corresponden a mutaciones germinales en APC (Adenomatous polyposis coli), un gen supresor de tumores, y genes de reparación de ADN como genes mismatch repair (MMR), respectivamente [4].

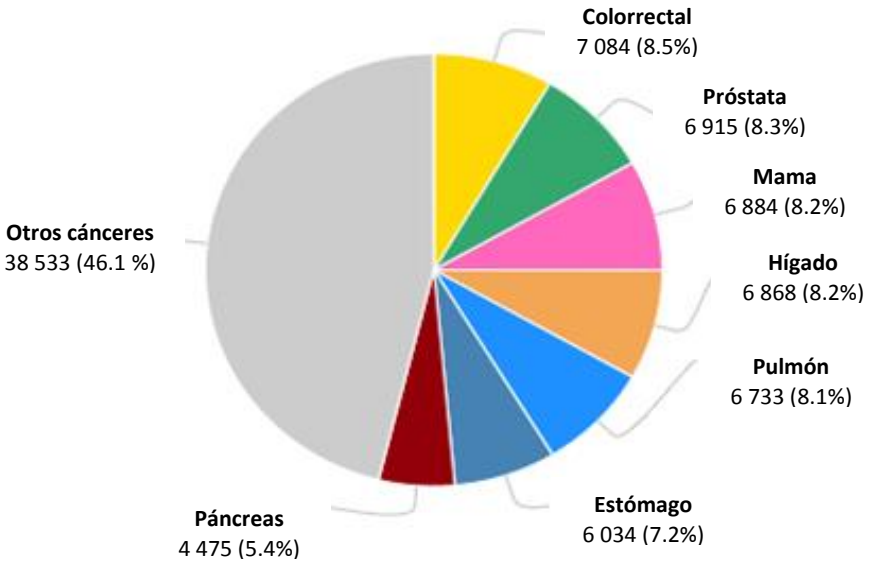
Actualmente se ha registrado un incremento en la incidencia del CCR, en México el CCR es el tercer cáncer más frecuente y el primero en mortalidad en ambos sexos, seguido del cáncer de próstata y cáncer de mama (**Fig1**) [3].

Número estimado de nuevos casos en 2018 en México de; todos los cánceres, ambos sexos, todas las edades



Total: 190 667

Número estimado de muertes en 2018 en México de; todos los cánceres, ambos sexos, todas las edades



Total: 83 476

Fig 1. Número estimado de incidencia y mortalidad de todos los cánceres en México en ambos sexos: GLOBOCAN 2018.

El cáncer de colon asociado a colitis (CAC) es un subtipo de CCR el cual es desarrollado por una respuesta inflamatoria previa en el colon. Pacientes con enfermedades inflamatorias intestinales (IBD) como la enfermedad de Crohn (CD) y la colitis ulcerativa (CU) tienen entre 10-20% mayor de probabilidad de desarrollar CCR. Estudios epidemiológicos muestran que la CU incrementa hasta un 20% la probabilidad de desarrollar CCR a diferencia de la CD con 8% de riesgo después de 30 años de haber sido diagnosticada la IBD [5]. En el caso del CAC, la mortalidad de los pacientes es de hasta un 50%, mucho mayor que los pacientes con CCR asociado a factores hereditarios [6,7].

Inflamación en CAC

Actualmente, el CAC representa el mejor ejemplo del proceso patológico: inflamación-displasia-cáncer [5]. En la colitis ulcerativa son características las lesiones profundas en la capa de la mucosa en donde la inflamación crónica contribuye a la tumorigénesis a través del daño al ADN por el incremento del estrés oxidativo en el colon, así como en la proliferación, crecimiento y evasión de la apoptosis de las células epiteliales (IEC) pre-transformadas [8].

En este proceso, el estrés oxidativo es principalmente producido por células inflamatorias del sistema inmune como macrófagos, monocitos y neutrófilos, que son una fuente importante de especies oxígeno reactivas (ROS) e intermediarios de nitrógenos reactivos (RNI). Existen diferentes tipos de daño oxidativo al ADN entre los que se han reportado: ruptura del esqueleto azúcar fosfato de una o de las 2 hebras, modificación de las bases nitrogenadas (saturación y fragmentación del anillo de timina) y la formación de uniones cruzadas (*cross-links*) ADN-ADN ó ADN-proteína, generando: modificación de las bases de ADN, generación de sitios apuricos, ruptura de una cadena del ADN, mutaciones, activación de oncogenes e inactivación de genes supresores y daño endotelial que favorece la metástasis[9].

Además, en la inflamación crónica en el colon hay un incremento en los niveles de citocinas inflamatorias con capacidad oncogénica como TNF-α, IL-1β, IL-17 e IL-6. Estas citocinas influyen en el proceso de carcinogénesis a través de la activación de vías de señalización asociadas con proliferación y evasión de la apoptosis como NF-κB, STAT3, señalización mediada por AKT y β-catenina, entre otras [8,10,11].

Señalización de NF-κB

El factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-κB) consiste en cinco diferentes proteínas de unión al ADN que forman una variedad de homodímeros y heterodímeros. NF-κB es un regulador clave de respuestas inmune innatas y adaptativas que además puede acelerar la proliferación celular, inhibir la apoptosis, promover migración celular e invasividad y estimular la angiogénesis y metástasis [12].

Hay dos tipos de la vía de señalización de NF-κB: la vía clásica (vía canónica) y la alternativa (no canónica). La vía clásica es rápidamente activada por citocinas inflamatorias como TNF-α e IL-1β, PAMPs y DAMPs que activan receptores específicos y moléculas adaptadoras. La activación de la vía alternativa es lenta y depende de la síntesis *de novo* de la quinasa inductora de NF-κB (NIK o MAP3K14) y es activada por linfotoxina (LT), TNFSF11, ligando de CD40 y BAFF [13].

La vía clásica o canónica posee los dímeros p50/p65 (codificados por RELA) unidos a su inhibidor IκB-α. Una vez iniciado el estímulo para la activación de NF-κB, IκB-α unido a NF-κB es fosforilado por el complejo IKK que comprende las subunidades catalíticas IKKα, IKKβ y la subunidad reguladora IKKγ (también conocida como NEMO), que sirve como centro de varias entradas de activación de NF-κB. La degradación de IκB-α permite a NF-κB translocarse al núcleo y mediar la transcripción de varios genes diana tales como citocinas inflamatorias (TNF-α, IL-1, IL-6), factores de crecimiento (factor estimulador de colonia de macrófagos y granulocitos, GM-CSF) quimiocinas y sus ligandos (CXCL1, CCL3, CXCL2, CCL2 y CCL5), metaloproteasas (MMP9), proteínas proliferativas (ciclina D1), proteínas anti-apoptoticas (BCL-X_L, BCL-2 y FLIP), enzimas proinflamatorias (COX2 e iNOS), factores

angiogénicos (VEFG), moléculas de adhesión (VCAM1, ICAM-1 y E-selectina) e inhibidores de la señalización de NF-κB (IκBα y A20) [14] **(Fig 2)**.

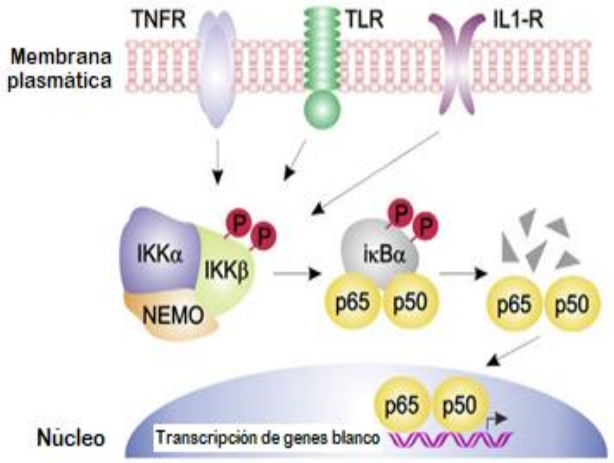


Fig 2. Activación canónica de la vía de señalización de NF-κB (Esquema tomado y modificado de Espín-Palazón *et al.,* 2016).

En las células cancerígenas, NF-κB incrementa la expresión de la telomerasa a nivel transcripcional y a su vez, la telomerasa se une a p65 para mejorar la transcripción de TNF-α, IL-6 y otros blancos dependientes de NF-κB, estableciendo así una retroalimentación [15].

En CAC, el papel oncogénico de NF-κB en IECs es mediado a través de funciones anti-apoptoticas las cuales previenen la eliminación de las células premalignas. Por otro lado, la activación de NF-κB en células del sistema inmune como células mieloides y macrófagos de la lámina propia, estimulan la proliferación de las células IEC premalignas y malignas a través de la secreción de citocinas y factores de crecimiento. La clara retroalimentación entre ambos tipos celulares promueve la carcinogénesis en diferentes etapas. Incluso, estudios recientes sugieren quepacientes con resistencia a terapias anti-tumorales muestran una mayor activación de NF-κB por lo que las IEC´s tumorales son resistentes a la apoptosis [14] [17].

Vía de señalización de STAT3

El transductor de señal y activador de transcripción 3 (STAT3) es un miembro de siete proteínas (STATs 1, 2, 3, 4, 5a, 5b y 6). STAT3 modula la transcripción de genes implicados en la regulación de una variedad de funciones críticas, incluyendo proliferación, diferenciación celular, apoptosis, angiogénesis, metástasis y respuesta inmune [18].

La estructura de STAT3 está compuesta por: un dominio de dimerización de STAT, un dominio *coiled-coil* involucrado en las interacciones proteína-proteína; un dominio de unión al ADN, un dominio de homología 2 Src (SH-2) el cual sirve para la unión de STAT al receptor de la citocina fosforilada y para la activación de STAT3, y un dominio de transactivación C-terminal **(Fig 3)**. La vía de señalización de STAT3 puede ser activada por citocinas tales como IL-6, IL-10, IL-27, LIF y hormonas de crecimiento [19]

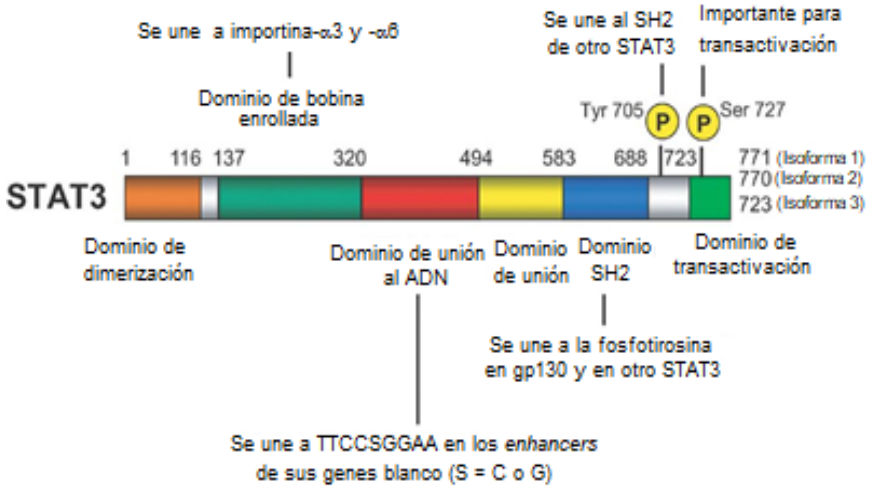


Fig 3. Estructura de STAT3 (figura tomada y modificada de Hirai, H. *et al.,* 2012).

En CAC, la citocina pleiotrópica IL-6 es un potente activador de la vía de señalización de STAT3. La señalización de IL-6/STAT3 es iniciada por la unión de IL-6 a su receptor unido a membrana celular (IL-6R) expresado principalmente en hepatocitos y leucocitos o en forma soluble (sIL-6R). El sIL-6R puede ser generado por dos mecanismos: rompimiento mediado por proteasas de la forma del IL-6R unido a la membrana o por *splicing* alternativo del ARN mensajero de IL-6R, que da lugar a una variante que carece del dominio transmembranal.

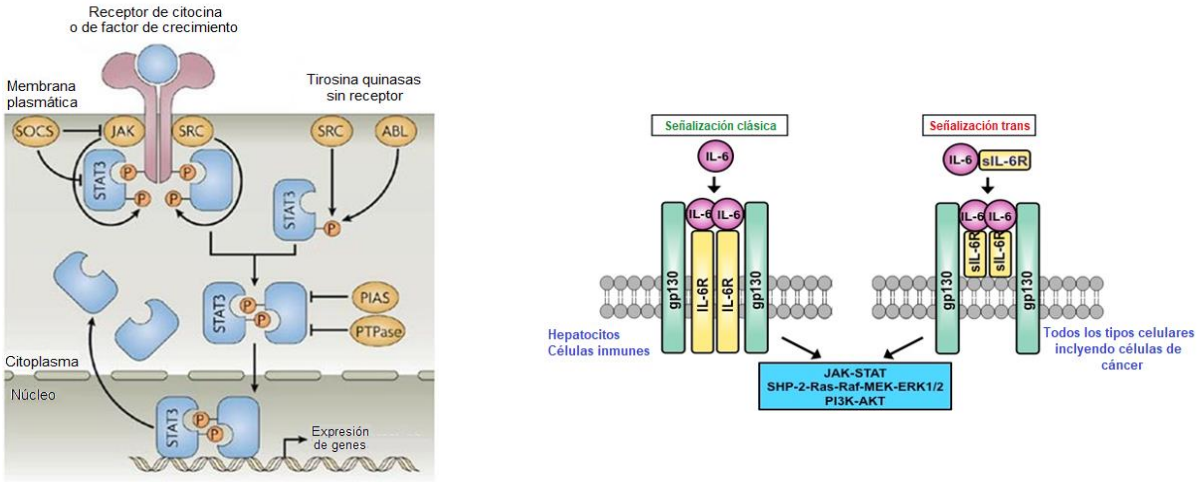


Fig 4. Vía de señalización de STAT3 y estructura del complejo IL-6/IL-6R/gp 130 (Esquema y figura modificadas de Yu, H. *et al*., 2007 y Heo, T. *et al.,* 2016).

La unión de IL-6 a su receptor induce el reclutamiento y homodimerización de dos subunidades de gp130, lo que favorece la transactivación de la proteína JAK[21]. Una vez activada JAK, esta quinasa fosforila un residuo de tirosina en la región citoplasmática del receptor que sirve como sitio de unión para STAT3. STAT3 es reclutada al receptor y fosforilada por las JAK, posteriormente, STAT3 se disocia del receptor para generar homodimerosen donde el dominio SH-2 de cada monómero interactúa con el residuo Y705 de cada STAT3. Los dímeros de STAT3 se translocan al núcleo, donde se unen a elementos específicos de respuesta del ADN en las regiones promotoras de los genes diana para regular su transcripción [18,22]. Este factor de transcripción es constitutivamente activado en células tumorales y células del sistema inmune y juega un papel importante en la carcinogénesis [23]. Por otro lado, reguladores negativos de STAT3 como SOCS3 (supresor de señalización de citocinas), SHP2 (proteína tirosina-fosfatasa 2) y PIAS3 (inhibidor de proteína de STAT3 activado) son raramente mutados en tumores sólidos [24]. Las principales vías que contribuyen a la activación de STAT3 en cáncer son el aumento de la secreción de citocinas y factores de crecimiento en el microambiente tumoral, así como la sobreexpresión de las proteínas tirosina quinasas y la modulación epigenética de los reguladores negativos de STAT3 [23].

En CAC, la vía IL-6/STAT3 es uno de los principales reguladores de la supervivencia y proliferación de las IEC pre malignas y malignas a través de la expresión de c-Myc, Mcl-1, ciclina D1 y BCL-2. También, está implicada en la promoción de invasión y metástasis, modulación de los subtipos de células T protumorales y regulación del infiltrado mieloide celular, por lo que la vía IL-6/STAT3 es un crucial promotor de tumor en CAC.

La IL-6 además de activar a la vía de señalización de STAT3, también puede activar a vías de señalización como Ras/ERK (quinasa reguladora de la señal extracelular) y PI3K/AKT (**Fig 4)** [22,25,26]

Vía de señalización de PI3K/AKT

La vía de la PI3K (fosfatidilinositol-3 quinasa) es activada por el estímulo de receptores de membrana con actividad de tirosina quinasa, los cuales fosforilan el sustrato del receptor de insulina (IRS); éste último, a la vez, fosforilará la subunidad p85 de la PI3K.

Otros mediadores inflamatorios como IL-6, IL-1, TNF-α y LPS también pueden inducir la activación de PI3K/AKT a través de sus respectivos receptores. Una vez fosforilada la subunidad p85, se genera un cambio conformacional de PI3K que conduce a la unión de la subunidad catalítica p110. La PI3K activada fosforila el fosfatidilinositol bifosfato (PIP2) convirtiéndolo a fosfatidilinositol trifosfato (PIP3). AKT se une a PIP3 en la membrana plasmática, permitiendo a PDK1 acceder y fosforilar a AKT en el dominio de T308 conduciendo a la activación parcial de AKT. Por otro lado, mTORC2 fosforila a AKT en el dominio de la serina 475 estimulando la completa actividad de AKT. AKT es desfosforilada en T308 por la proteína fosfatasa 2A (PP2A) y por la proteína fosfatasa que contiene dominios repetidos ricos de leucina PH (PHLPP1/2) en S475. Además del supresor de tumor y homólogo de tensina (PTEN) el cual inhibe la actividad de AKT por la desfosforilación de PIP3 [29]

Una vez activada, AKT conduce a eventos de fosforilación específica de sustrato en citoplasma y en núcleo, incluyendo la fosforilación para la inhibición de la proteína pro-apoptótica FOXO, inactivación de varios factores pro-apoptoticos como BAD, procaspasa 9 y

factores de transcripción como FKHR (*forkhead)*. También, AKT fosforila e inactiva al substrato de AKT rico en prolina de 40 kDa (PRAS40) y la proteína 2 de esclerosis tuberus (TSC2) activando a mTORC1, que actúa sobre la síntesis de proteína vía activación de S6K1, el cual fosforila a la proteína ribosomal S6, promoviendo la síntesis de proteína y proliferación celular [30]. De igual forma, AKT induce la fosforilación e inactivación de GSK3β que participa en la vía de señalización Wnt/β-catenina **(Fig 5)** [31].

Otros factores de transcripción activados por AKT que incrementan la expresión de genes anti-apoptóticos incluyen a NF-*κ*B y HIF-1α. La activación de NF-*κ*B por AKT se genera por la fosforilación en T23 de la subunidad IKKα permitiendo la fosforilación de IκBα, así su subsecuente degradación deja libre a NF-*κ*B favoreciendo su translocación al núcleo [32].

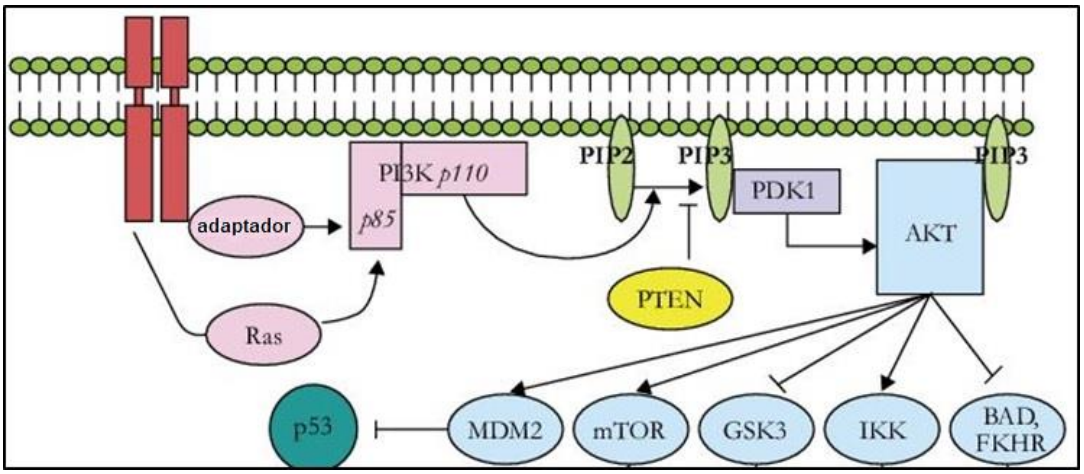


Fig 5. Vía de señalización de PI3K/AKT (esquema tomado y modificado de Alexis Cortot *et al*. 2006).

Vía de señalización mediada por β-catenina

La vía canónica de Wnt/β-catenina es activada cuando los ligandos de Wnt se unen a la región del receptor transmembranal *Frizzled* (Fz) y su co-receptor proteína 6 asociada a receptor de lipoproteina (LRP6) o a LRP5. La formación del complejo Wnt-Fz-LRP6 con el reclutamiento de la proteína *Dishevelled* (Dvl) resulta en la fosforilación de LRP6, activación y reclutamiento del complejo Axina a los receptores. Estos eventos conducen a la inhibición de la fosforilación de β-catenina y por lo tanto su acumulación en el citoplasma y la

subsecuente translocación al núcleo para formar el complejo con TCF/LEF y activar la expresión de genes blanco de Wnt[33].

En ausencia de Wnt, la proteína β-catenina citoplasmática es constantemente degradada por la acción del complejo Axina, el cual está compuesto por; la proteína Axina, el producto del gen coli poliposis adenomatosa (APC), Quinasa 1 de caseína (CK1), y la quinasa sintasa de glicógeno (GSK3β). CK1 y GSK3 fosforilan la región amino terminal de β-catenina, la β-catenina fosforilada es reconocida por β-Trcp, una subunidad ligasa de E3 ubiquitina, posteriormente es ubiquitinisada y degradada vía proteosoma [34]. La eliminación constante de β-catenina previene su acumulación nuclear e interacción con el complejo TCF/LEF.

En CCR asociado a factores hereditarios se ha observado hasta un 90% en mutaciones en el gen APC, lo que conlleva a la acumulación nuclear de β-catenina [10]. En contraste, en etapas tempranas de CAC se observa la ausencia de mutaciones en APC, sin embargo, la constante acumulación de β-catenina nuclear es reportada. En CAC, mediadores solubles contenidos en el microambiente pueden inducir la activación de AKT, NF-κB y ERK, su señalización induce la acumulación de β-catenina, su interacción con el complejo TCF/LEF y transcripción de genes asociados a proliferación celular como c-Myc. También se han reportado mutaciones en el residuo serina y treonina de β-catenina, generando β-catenina mutada que escapa de su fosforilación y degradación [35].

Infecciones por parásitos helmintos y cáncer

Diversos factores ambientales (químicos, físicos y biológicos) influyen claramente en la iniciación, promoción y progresión del cáncer. En el caso de los factores biológicos, se ha demostrado que las infecciones causadas por algunas bacterias, virus, protozoos y parásitos helmintos están relacionadas con la carcinogénesis, por ejemplo el virus de papiloma humano y cáncer cervical, *Helicobacter pylori* y cáncer gástrico. Según la IARC, tres parásitos helmintos son ampliamente aceptados como carcinógenos para los humanos, tal es el caso de la infección por *Schistosoma haematobium* asociada con el cáncer de vejiga, *Clonorchis sinensis* y *Opisthorchis viverrini*, los cuales son trematodos asociados al

desarrollo de colangiocarcinoma del hígado [36]. El potencial carcinógeno de estos parásitos helmintos radica en la activación de los oncogenes, la inactivación de los genes supresores de tumores, las mutaciones somáticas como factores clave en el inicio y la promoción de la malignidad. Por ejemplo, durante la opistoriasis y la esquistosomiasis urogenital, la producción de metabolitos reactivos de los precursores parecidos a los de oxysterol y de tipo estrógeno del helminto, representan genotoxinas que mutan los genes de las células epiteliales que recubren el tracto biliar y la vejiga urinaria, inciando el cáncer del conducto biliar y el carcinoma de células escamosas de la vejiga [37]. Además de los metabolitos específicos del helminto, se sugiere que las infecciones por helmintos inducen una reparación excesiva y desarrollo de la angiogénesis. Un estudio demostró a su vez que la infección con *O. viverrini* provocó cambios en las comunidades microbianas del gastrointestinal, incluida la aparición de microrganismos en el sistema biliar [38].

Las regiones del mundo con mayores tasas de cáncer de colon son aquellas asociadas con altos estándares de vida, como América del Norte, Europa (Norte), Australia y Nueva Zelanda [3], donde la tasa de incidencia de enfermedades parasitarias, especialmente infecciones por helmintos, es baja [39]. Como mencionamos, varios parásitos helmintos han sido identificados como promotores de algunas neoplasias, mientras que otros han sido reportados como reguladores negativos del desarrollo del cáncer [40]. Aunque múltiples mecanismos están involucrados en la modulación del desarrollo del cáncer, no se han descrito completamente.

Regulación del sistema inmune por parásitos helmintos: *Taenia crassiceps*

Los parásitos helmintos constituyen un grupo de metazoarios muy exitosos en la colonización de sus hospederos debido a que han logrado desarrollar un número único de adaptaciones que impiden su expulsión. A pesar de su gran tamaño, de su migración local o sistémica a través del cuerpo, los helmintos únicamente provocan inflamación limitada en el tejido invadido, y promueven un ambiente inmunoregulador el cual asegura su supervivencia [41]. Tales adaptaciones son atribuidas a su larga coevolución con su hospedero, lo que les

permite realizar una modulación efectiva del sistema inmune. Se han identificado varios mecanismos mediante los cuales los helmintos restringen las respuestas inmunes del hospedero: la polarización de la respuesta inmune hacia un perfil Th2 caracterizado por la secreción de citocinas anti-inflamatorias como IL-4 IL-5, IL-10, IL-13, anticuerpos IgE, IgG1 e IgG4 e inducción de macrófagos activados alternativamente (AAMs) [41].

La exposición a agentes infecciosos evoca un efecto fundamental sobre el desarrollo y comportamiento del sistema inmune. La hipótesis de la higiene sugiere una relación inversa entre infecciones parasitarias e hiperreactividad del sistema inmunológico. Por ejemplo, los estudios epidemiológicos de áreas endémicas de parásitos indican que la experiencia prenatal o temprana de las infecciones puede afectar la reactividad inmunológica de un individuo [39].

Sin embargo, la capacidad de los helmintos para amortiguar la patología en enfermedades inflamatorias establecidas tales como diabetes e IBD, implica que pueden tener efectos terapéuticos incluso si el sistema inmunológico se ha desarrollado en un entorno de baja infección [39,42,43]. En nuestro grupo de investigación, hemos reportado el potencial terapéutico de *Taenia crassiceps* y sus productos de excreción/secreción (TcES) en enfermedades autoinmunes e inflamatorias.

Taenia crassiceps es un parásito helminto que pertenece a la clase cestoda. En su ciclo de vida, el estadio adulto de *T. crassiceps* se aloja en el intestino de los cánidos los cuales son su huésped definitivo, los huevos infectivos son expulsados a través de las heces de los cánidos los cuales son ingeridos por roedores siendo estos huéspedes intermediarios. En los roedores, los huevos infectivos a traviesan el epitelio intestinal, alojándose y diferenciándose a larva o cisticerco. Los roedores infectados son ingeridos por cánidos, cerrando de esta manera el ciclo [44].

Una característica de los cisticercos de *T. crassiceps* es su capacidad de reproducirse asexualmente a través de gemación, permitiéndole permanecer en su hospedero por largos periodos de tiempo. Experimentalmente, la inoculación intraperitoneal de metacestodos de *T. crassiceps* en roedores permite su mantenimiento en el laboratorio. Este modelo de infección experimental ha servido para comprender diferentes fenómenos asociados a su

capacidad inmunoreguladora [45–48], por ejemplo, la infección crónica con *T. crassiceps* induce una respuesta inmune dominante de tipo Th2 rica en IL-4, IL-13, anticuerpos IgG1 e IgE y AAMs además de ser dependiente de la señalización por STAT6 [44]. Los AAMs generados tras la infección por *T. crassiceps* muestran menor secreción de IL-1β, IL-12 y ON, además expresan altos niveles de Arginasa 1 (Arg1), Ym1, FIZZ, receptor de manosa (MMR), cadena alfa del receptor de interlecina 4 (IL-4Rα), PD-L1 y PD-L2 [49].

En modelos *in vivo* de enfermedades autoinmunes e inflamatorias tales como EAE (encefalomelitis autoinmune experimental), CU y diabetes tipo 1 (DT1), la infección con *T. crassiceps* genera protección del curso de la enfermedad con diferentes posibles mecanismos, por ejemplo, en EAE, la infección con *T. crassiceps* induce la disminución de citocinas inflamatorias como IFN-γ, TNF-α, IL-17 y reducción en el reclutamiento de infiltrado inflamatorio a la medula espinal [50]. En DT1 la infección por *Taenia* reduce los niveles de glucosa en sangre y el infiltrado de leucocitos al páncreas, este efecto protector fue asociado a altos niveles sistémicos de IL-4 y reducción de TNF-α [47]. En colitis aguda experimental, la infección por *T. crassiceps* reduce la inflamación en colon, disminuyendo el daño histopatológico característico de la enfermedad [51]

Es ampliamente aceptado que la inhibición de las respuestas proinflamatorias y la inducción de la respuesta inmune Th2 durante las infecciones por helmintos dependen de la capacidad del parásito para excretar/secretar antígenos [44]. *In vitro*, los productos excretados/secretados (TcES) que libera el metacestodo de Taenia y cuya composición aún es desconocida, tienen la capacidad de interferir con la maduración de las células dendríticas (DC), estas células se vuelven refractarias al estímulo con LPS induciendo bajos niveles de las citocinas inflamatorias IL-12, IL-5 y TNF-α. Además, las DCi (células dendríticas inmaduras) generadas tras el estímulo por TcES inducen la diferenciación de células T $CD4^+$ vírgenes a Th2 [48]. Reportes generados por nuestro grupo de trabajo indican que en modelos *in vivo* de EAE, DT1 y colitis aguda experimental los TcES son capaces de mimetizar algunas de las características inducidas por la infección con *Taenia crassiceps,* como el reclutamiento de AAMs e inducción de citocinas anti-inflamatorias.

Considerando la capacidad inmunoreguladora de *T. crassiceps* y sus antígenos, en este trabajo de tesis se estudió el efecto de la infección y de los Tces en el curso del cáncer de colon asociado a colitis y los mecanismos en lo que subyace el mismo.

HIPÓTESIS

La infección y los productos excretados/secretados por *Taenia crassiceps* modularán negativamente diferentes procesos inflamatorios que favorecen el desarrollo de cáncer de colon asociado a colitis.

OBJETIVO GENERAL

Evaluar el efecto de la infección y los productos excretados/secretados de *Taenia crassiceps* (TcES) durante el desarrollo de cáncer de colon asociado a colitis.

Objetivos particulares

- Evaluar el efecto de la infección previa de *T. crassiceps* en el desarrollo de CAC.
- Determinar el efecto de los productos excretados/secretados de *T. crassiceps* (TcES) durante el desarrollo del CAC.
- Determinar el o los posibles mecanismos de acción de los TcEs en el desarrollo del CAC.

MATERIALES Y MÉTODOS

Ratones

Ratones hembras BALB/c y C57BL/6 de ocho a diez semanas de edad de Harlan Laboratories (México) y se mantuvieron en un ambiente libre de patógenos en las instalaciones de animales de la Facultad de Estudios Superiores-Iztacala (FESI), Universidad Nacional Autónoma de México (UNAM). Los animales se alimentaron con Purina Diet 5015 (Purina) y agua *ad libitum*. Todos los procedimientos experimentales se ajustaron estrictamente a las recomendaciones de la Guía para el cuidado y uso de animales de laboratorio de los Institutos Nacionales de la Salud (EE.UU.) Y fueron aprobados por el Comité de Ética de los Experimentos con Animales de la FES-Iztacala (UNAM).

Infección con *T. crassiceps* y obtención de productos TcES

Infección con *T.* crassiceps: Ratones hembras BALB/c fueron inoculados con 20 metacestodos de *T. crassiceps* (cepa ORF) vía intraperitoneal 6 semanas después fue inducido el CAC como se detalló anteriormente.

Obtención de TcES: Metacestodos de *T. crassiceps* (cepa ORF) se recolectaron en condiciones estériles de la cavidad peritoneal de ratones hembras BALB/c después de 8-10 semanas de infección. Los cisticercos obtenidos de la infección se cultivaron en solución salina fisiológica a 37° C durante 24 h. Los TcES se recuperaron del sobrenadante y se centrifugaron durante 10 min a 3500 rpm. A continuación, las proteínas de peso molecular superior a 50 kDa se concentraron utilizando una membrana de corte de 50 kDa Ultrafiltro Amicon (Millipore, Billerica, MA, EE. UU.). Se recolectaron las moléculas de alto peso molecular y se agregaron inhibidores de proteasas. La concentración de proteína se determinó utilizando el ensayo de Bradford y el TcES se almacenó a -70° C hasta su uso posterior. Se evaluaron los niveles de endotoxinas que fueron no detectables de los productos excretados/secretados por *T. crassiceps* (<0.4 EU / mg) utilizando el kit de E-Toxate (Sigma, EE. UU.)

Desarrollo de la Colitis Ulcerativa

En agua para beber estéril fue disuelto DSS (MW: 35,000–50,000; MP Biomedicals, Solon, USA) al 4% y administrado *ad libitum* por 7 a 10 días.

Desarrollo del cáncer de colon Asociado a colitis

Se desarrolló un modelo de CAC ampliamente utilizado como se describió anteriormente [52]. Brevemente, los ratones recibieron una inyección intraperitoneal (*i.p.*) con 12.5 mg/kg de azoximetano (AOM, Sigma, EE. UU.). Cinco días después se administró sulfato de dextran sódico (DSS, MW: 40 000, Alfa, Canadá) al 2% en agua para beber estéril durante 7 días consecutivos. Posteriormente, los ratones se mantuvieron con agua regular durante 14 días y se sometieron a dos ciclos más de tratamiento con DSS. En el día 26 de la inducción de CAC, los productos excretados/secretados por *Taenia crassiceps* (TcES) fueron inoculados vía intraperitonealtres veces por semana (200 µg por ratón) hasta finalizar la inducción de CAC. Todos los ratones se sacrificaron el día 68, después de lo cual se extrajo el colon, se pesó y se sometió a inspección macroscópica y examen histopatológico.

Cultivo de macrófagos y medición del óxido nítrico.

Se cultivaron suspensiones unicelulares de médula ósea aisladas de fémures y tibias de ratones sin tratamiento previo, en RPMI suplementado con suero de bovino fetal (SBF) al 10%, penicilina y estreptomicina. Se añadió una concentración de 10 ng ml − 1 M-CSF (R&D systems, Oxon, Reino Unido) los días 0, 2 y 4 a los cultivos de médula ósea. A los 7 días los cultivos fueron estimulamos con 25 µg de TcES por 5 minutos, posteriormente fue agregado LPS (1 µg/ml) y cultivamos a 37° C con 5% de CO_2. Los sobrenadantes se colectaron 24 h más tarde y la concentración de óxido nítrico fue determinada utilizando el reactivo de Griess de acuerdo con las instrucciones del fabricante.

Análisis histológico

Para el análisis histológico, las secciones longitudinales del intestino grueso de los ratones se fijaron inmediatamente mediante inmersión en paraformaldehído al 4%, después de un día el tejido se deshidrató en gradiente de concentración de alcohol para la posterior inclusión de parafina y el análisis histológico. Se examinaron los tejidos de colon utilizando 5 µm de espesor y se tiñeron con hematoxilina y eosina para visualizar la morfología o con la tinción con azul de Alcian para visualizar las células caliciformes, utilizando un microscopio óptico (Axio Vert.A1, Carl Zeiss). Para inmunohistoquímica e inmunofluorescencia, las secciones de colon se incubaron toda la noche a 4° C con anticuerpo primario para: p-STAT3, p-AKTSer473, PTEN, β-catenina activa, ciclina D1, c-Myc (Cell Signaling Technology, EE. UU.), Ki67, DNMT1, Ly6G (Abcam, USA), β-catenina, 8-Hydroxyguanosina (GeneTex, USA), BCL-2 (Biolegend, USA), con sus respectivos anticuerpos secundarios durante 1 h. Los análisis confocales se realizaron utilizando un sistema de microscopía confocal Leica TCS SP8 (Leica).

Cultivo de colon

Se cortaron secciones de 0.5 cm de la zona distal del colon distal y se lavaron tres veces con medio RPMI con penicilina y estreptomicina al 2%. Los fragmentos de colon se colocaron en un cultivo de placa de 24 pozos en medio RPMI con penicilina y estreptomicina al 1% y se estimularon o no con LPS (1µg/mL) por 24 h a 37°C con 5% de CO_2. Los sobrenadantes se recolectaron 24 h más tarde y se determinó la concentración de citocinas.

Cultivo de células del bazo

Los esplenocitos se extrajeron mediante perfusión de bazo con 10 ml de solución salina estéril utilizando agujas 25G en una placa de Petri. Después de la lisis de eritrocitos, las células se lavaron en la misma solución tres veces antes de la resuspensión en medio RPMI suplementado con SBF, penicilina y estreptomicina. Las células se colocaron en placas de 24 pozos (Falcon; BD Labware, Mountain View, CA) en un volumen final de 300 µl de medio.

Los cultivos se mantuvieron durante 48 h antes de colectar los sobrenadantes, posteriormente fueron almacenados a -70°C.

Detección de citocinas

La producción de las citocinas IL-17F, IL-23, IL-31 y la molécula de adhesión ICAM1 en extractos de proteína de colon se cuantificó utilizando Bio-Plex MAGPIX (BIO-RAD, EE. UU.). Los niveles de IL-1β, TNF-α, IL-10, IL-6, L-4, IL-17A e IL-17E se cuantificaron para el método ELISA en cultivo de colon y sobrenadantes de bazo (Peprotech México, Ciudad de México, México), ambos según los protocolos del fabricante.

Purificación de macrófagos peritoneales, transferencia adoptiva y ensayo de supresión *in vitro*.

Las células de exudado peritoneal se aislaron de las cavidades peritoneales de ratones infectados con *T. crassiceps* 8 semanas después de la infección. Los receptores Fc fueron bloqueados a través de la incubación de las células con suero durante 10 min a 4 ° C.

Luego, las células se marcaron con APC anti-F4/80 y receptor de anti-manosa (células FITC; 0.25 ug / 106; Biolegend) a 4°C durante 20 min. Las poblaciones $F480^{+}$ MR^{+} y $F480^{+}$ MR^{-} aisladas utilizando un citómetro de flujo FACs Aria III. La viabilidad de las células fue del 90% y la pureza del 95%. Un millón de células $F4/80^{+}MR^{+}$ o $F4/80^{+}MR^{-}$ purificadas se inyectaron *i.p.* en ratones BALB/c 2 días después del inicio del tratamiento con DSS. De manera independiente, también fueron evaluadas las propiedades antiproliferativas de las células $F4/80^{+}MR^{+}$ o $F4/80^{+}MR^{-}$ obtenidas de la infección por Taenia sobre linfocitos T. Esplenocitos de ratones no infectados fueron colocados en placas de 96 pozos previamente estimuladas con anti-CD3/CD28 (2 µg/mL), después de 4 h, se adicionaron células $F4/80^{+}MR^{+}$ o $F4/80^{+}$ MR^{-} en diferentes proporciones. La proliferación se evaluó después de 72 h mediante un ensayo de dilución CFSE utilizando un citómetro FACSCalibur

Extracción de ARN y RT-PCR

El ARN total del tejido del colon se extrajo utilizando un kit de purificación de ARN/proteína (Norgen Biotek Corp, Canadá). La concentración de ARN se determinó midiendo la absorbancia a 260 nm. Se utilizó un microgramo de ARN total para la síntesis de ADNc de primera cadena con el kit de síntesis de ADNc de primera cadena RevertAid H Minus (Thermo Fisher Scientific). Las secuencias de los cebadores y las condiciones de los ciclos se enumeran en la Tabla 1.

Gen	Secuencia	Temperatura de fusión
GAPDH	F-TCGGTGTGAACGGATTTGGC R-CTCTTGCTCAGTGTCCTTGC	56° C
Arg-1	F-CAG AAG AAT GGA AGA GTC AG R-CAG ATA TGC AGG GAG TCA CC	54° C
Ym1	F-TCACAGGTCTGGCAATTCTTCTG R-TTTGTCCTTAGGAGGGCTTCCTC	56° C
FIZZ	F-GGTCCCAGTGCATATGGATGAGACCATAG R-CACCTCTTCACTCGAGGGACAGTTGGCAGC	65° C
iNOS	F-GCCACCAACAATGGCAACAT R-AAGACCAGAGGCAGCACATC	60° C
IL-6R	F-GCCCAACACCCAAGTCAACT R-TATAGGAAACAGCGGGTTGG	58° C
CXCR2	F-AGCAAACACCTCTACTACCCTCTA R-GGGCTGCATCAATTCAAATACCA	58° C
TLR4	F-GTTGCAGAAAATGCCAGGATG R-CAGGGATTCAAGCTTCCTGGT	58° C

Tabla1. Secuencias de cebadores para PCR punto final y qPCR

Evaluación de células T reguladoras y monocitos por citometría de flujo.

Para el análisis de células T reguladoras, se obtuvieron suspensiones de células de bazo y PECs de ratones reporteros C.CgFoxp3tm1Tch/J las cuales fueron teñidas con α-CD4 y α-CD25 (Biolegend), para su análisis fue seleccionada la población de células $CD4^+$. De esta población se analizaron las expresiones de CD25 y FOXP3 utilizando el sistema FACsAria Fusion flow cytometer (BD Biosciences).

Para la citometría de flujo de sangre circulante y células de lámina propia, al finalizar la inducción de colitis y en el modelo de CAC se obtuvo sangre de circulación en el día 12, 33 y 54. Las células se lavaron con PBS 1X y se bloquearon utilizando anticuerpos contra CD16/CD32. Las células se tiñeron simultáneamente con anticuerpos contra CD11b, Ly6C, Ly6G (BioLegend) y CCR-2 (R&D Systems) durante 30 min a temperatura ambiente. Para aislar las células de la lámina propia del colon, se lavó el colon con PBS frío, se abrió longitudinalmente y se cortó en trozos de 0.5 cm. Las células epiteliales y el moco se eliminaron mediante incubación de 30 minutos con HBSS que contenía FBS al 2%, EDTA 2 mM, a 37 ° C y agitación a 50 g. Las piezas de colon fueron digeridas en DMEM con 2 mg/ml de colagenasa VIII (Sigma) y 40 µg/ml de ADNasa I (Invitrogen) durante 2 horas a 37°C con agitación a 250 rpm. La suspensión de células digeridas se lavó a continuación con DMEM con FBS al 10%, se pasó secuencialmente a través de filtros de células de 100 y 40 µm, y se sedimentó por centrifugación a 448g durante 10 min. Las células se separaron posteriormente mediante centrifugación a través de gradientes de Percoll. Finalmente, se agruparon las células de los mismos grupos para su análisis. Los análisis de las células se realizaron utilizando el sistema FACSCalibur y el software Cell Quest (Becton Dickinson).

Supresión *in vivo* de la prostaglandina E2.

La producción de prostaglandina E2 fue bloqueada a través de la administración vía *i.p.* de indometacina (3 mg/kg). El grupo control se inyectaron con DMSO al 0.5% en un buffer de bicarbonato al 5% como control de vehículo

Línea celular (RKO) y cultivo celular en 3D.

Las células epiteliales de cáncer de colon de humano RKO se obtuvieron de *American Type Culture Collection*. Las células RKO se mantuvieron en medio esencial mínimo de Eagle (EE. UU.) suplementado con SBF al 10%, penicilina 50 U/ml y estreptomicina 50 µg/mL., y se cultivaron a 37ºC con 5% de CO_2. Posteriormente fueron tratadas con DMSO o 12,5 µg/ml de TcES durante 72 horas y estimuladas con 0.5 µg/ml de LPS durante 20 min. Posteriormente fue colectado el sobrenadante y centrifugando a 4000 rpm/min durante 10 minutos.

Para los cultivos en 3D, se colocaron aproximadamente 2 000 células sobre una membrana basal reconstituida (rBM) en placas de 16 pozos [53] y se cultivaron durante 10 días en presencia o ausencia de TcES. Las estructuras 3D se fijaron en paraformaldehído al 4% a temperatura ambiente durante 15 minutos. Las células se tiñeron con Oregon green-phalloidin and 4,6-diamidino-2-phenylindole (Invitrogen). Los análisis confocales se realizaron utilizando un sistema de microscopía confocal Leica TCS SP8 (Leica).

Análisis de proliferación y apoptosis de RKO.

La proliferación *in vitro* se midió sembrando 1 x 10^5 células en placas de cultivo celular de seis pozos y se estimularon con DMSO o TcES. En puntos de tiempo específicos, las células se tripsinizaron y se contaron utilizando el análisis de exclusión con azul tripán (Gibco).

El porcentaje de inhibición del crecimiento se calculó normalizando el tratamiento con TcES al control con DMSO. Los valores de CI50 se calcularon a partir de curvas de respuesta de dosis generadas en GraphPad PRISM utilizando un modelo de ajuste de curva de regresión no lineal.

La apoptosis se midió utilizando el kit de detección de apoptosis Annexin V-PE (BD PharMingen) seguido de citometría de flujo. Las células RKO (2 x 10^5) se sembraron en placas de cultivo celular de seis pozos y se trataron con vehículo o TcES durante 72 horas. Se recogieron células tanto flotantes como unidas, se lavaron dos veces con PBS frío y se suspendieron en PBS 1X. Se transfirió una parte alícuota de 100 µl de la suspensión celular (que representa 5×10^5 células) a un tubo de cultivo, al que se agregaron 5 µl de Anexina V-

PE y 5 µL de 7-aminoactinomicina D (7-AAD) la cual fue incubada durante 15 min a temperatura ambiente en obscuridad. El análisis de apoptosis se llevó a cabo utilizando un citómetro de flujo FACsAria Fusion (BD Biosciences). Se recolectaron un total de 10,000 células para cada muestra para análisis.

Ensayos de Western Blot

Las células RKO se lisaron en buffer con: Tris-HCl 50 mM, pH 8,0, NaCl 137 mM, glicerol al 10%, NP-40 al 1%, NaF 50 mM, fosfato de β-glicerol 10 mM, sodio 2 mM Ortovanadato, fluoruro de fenilmetilsulfonilo 1 mM y 10 µg/ml de aprotinina.

De tejido, la proteína total se extrajo de muestras de colon utilizando un kit de purificación de ARN/proteína (Norgen Biotek Corp, Thorold, ON, Canadá). Las concentraciones de proteínas de los cultivos celulares y de muestras de tejido de colon se determinaron mediante el ensayo de Bradford y se separaron mediante SDS-PAGE (se cargaron 30 µg de proteína en cada pozo) utilizando geles de acrilamida al 10%, seguido de transferencia a difluoruro de polivinilideno (PVDF) (Immobilon-P, Millipore, EE. UU.). Las membranas se bloquearon con leche sin grasa al 10% en PBS durante 2 h, los anticuerpos primarios de; β-actina SOCS3, PIAS3, fosfo-p65, p65 total, IκBα total, fosfo-IκBα (Cell Signaling Technology, USA) (Biolegend, USA) fueron incubados en leche al 5% en PBS durante toda la noche. La etapa de detección se realizó con IgG antirabbit acoplado a peroxidasa o IgG1 antimicrobiana (BioLegend, 1: 5000) durante 2 h. Los blots fueron revelados utilizando un sistema de detección ECL de acuerdo con las instrucciones del fabricante (Amersham, EE. UU.).

Análisis estadístico

Los datos se analizaron mediante un ANOVA de una vía seguido de la prueba de comparaciones múltiples de Tukey o mediante una prueba t de dos colas no pareada con GraphPad Prism 5 (San Diego, CA). Todas las pruebas estadísticas se realizaron considerando intervalos de confianza del 95%. Los datos se expresan como medias ± SE, donde * representa $p < 0.05$, ** representa $p < 0.01$ y *** $p < 0.001$.

RESULTADOS

El efecto regulador de parásitos helmintos y el desarrollo de diferentes tipos de cáncer es materia de discusión actualmente; en el presente trabajo se evaluó la respuesta reguladora de la infección previa por *Taenia crassiceps* y sus productos derivados en el desarrollo del CAC. En esta tesis se presentan los resultados en forma de artículos publicados (Apéndice I).

Artículo 1.

Ledesma-Soto Y, Callejas BE, Terrazas CA, Reyes JL, Espinoza-Jiménez A, González MI, León-Cabrera S, Morales R, Olguín JE, Saavedra R, Oghumu S, Satoskar AR, Terrazas LI. **Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins**. 2015. *Biomed Res Int.* 2015;2015:563425.

En este artículo se evaluó el efecto de la infección previa por *Taenia crassiceps* sobre el curso de la colitis aguda experimental.La infección previa con *T. crassiceps* redujo significativamente las manifestaciones de colitis inducida por DSS; como la pérdida de peso, el acortamiento de la longitud del colon, así como la gravedad del índice de actividad de la enfermedad, independientemente del fondo genético de los ratones (BALB/c o C57BL/6). En cuanto al perfil inmunológico, la pre-infección con *T. crassiceps* disminuyó los niveles sistémicos de citocinas proinflamatorias al tiempo que aumentaba los niveles de IL-4 e IL-10. A nivel local el infiltrado de células inflamatorias ($CD11b^{+}Ly6C^{hi}CCR2^{+}$) fue reducido notablemente. También, en tejidos de colon a través de ensayos de RT-PCR, los ratones infectados con *T. crassiceps,* mostraron una expresión incrementada de Arginasa-1 (un marcador ampliamente asociado a AAMs), pero una expresión disminuida de iNOS (marcador asociado a CAMs) en comparación con los ratones no infectados tratados con DSS. Con el propósito de identificar el posible mecanismo por el cual la previa infección con *Taenia* podría regular la respuesta inflamatoria en el modelo de colitis, decidimos evaluar el

porcentaje de células T reguladoras inducidas por la infección. Sin embargo, no observamos un incremento significativo de estas células por la presencia del parásito. Debido a que la infección por *Taenia* facilita la polarización de AAMs los cuales son asociados a reparación tisular, evaluamos su papel en la protección de la colitis a través de la transferencia adoptiva de AAMφ´s de ratones infectados a ratones cursando con colitis inducida por DSS, en donde encontramos que la transferencia temprana de AAMs redujo la gravedad de la inflamación del colon inducida por el DSS. Para confirmar el papel regulador de los AAMs generados tras la infección por el helminto en este modelo, decidimos bloquear la producción de PGE2 la cual es producida por AAMs mejorando también su polarización. Tras dosis diarias de indometacina bloqueamos la PGE2 en el grupo infectado y sin infección por *T. crassiceps* durante el curso de la colitis. Al finalizar la inducción de colitis observamos que la administración de indometacina eliminó las diferencias patológicas entre grupos, bloqueando el efecto regulador de *T. crassiceps* en el modelo de colitis por DSS. Finalmente, concluimos que la infección por *T. crassiceps* limita la patología de la colitis ulcerosa al suprimir las respuestas inflamatorias mediando la activación de AAMs.

Artículo 2.

León-Cabrera S, Callejas BE, Ledesma-Soto Y, Coronel J, Pérez-Plasencia C, Gutiérrez-Cirlos EB, Ávila-Moreno F, Rodríguez-Sosa M, Hernández-Pando R, Marquina-Castillo B, Chirino YI, Terrazas LI. **Extraintestinal Helminth Infection Reduces the Development of Colitis-Associated Tumorigenesis.** 2014. *Int J Biol Sci.* 10(9):948-56. doi: 10.7150/ijbs.9033.

En esta parte de la tesis evaluamos el papel de la infección por *Taenia crassiceps* en el curso del CAC. Para esto, infectamos a ratones Balb/c con metacestodos de *Taenia* en cavidad peritoneal, 6 semanas post-infección indujimos el CAC como se describió en la sección de materiales y métodos. Al finalizar la inducción de CAC, encontramos que el grupo previamente infectado con *T. crassiceps* presentó menor número de tumores en la zona media y distal del colon respecto al grupo CAC. También, en los ratones del grupo *T. crassiceps*+CAC se observó menor infiltrado de neutrófilos al colon y mantenimiento de células caliciformes productoras de mucina. En contraste, los ratones del grupo CAC mostraron una claro proceso de transformación de las células epiteliales, es decir; en tejido de colon observamos un gran número de figuras mitóticas y presencia de diversos pólipos con arreglos glandulares, reducción de células caliciformes y gran infiltrado celular al colon. También, β-catenina la cual es un marcador tumorigénico asociado a proliferación celular en cáncer, fue sobreexpresada en el colon de los ratones del grupo CAC en contraste al grupo *T. crassiceps*+CAC, lo que sugiere que estos últimos no desarrollaron tumorigenesis en colon.

Debido a que la infección por *T. crasssiceps* induce una respuesta inmune de tipo Th2 evaluamos la expresión de citocinas anti-inflamatorias y marcadores asociados a AAMs. En el tejido de colon del grupo *T. crass*+CAC encontramos una mayor expresión de la citocina IL-4 y una marcada disminución de IFN-γ, TNF-α y TGF-β. Además, la expresión de marcadores como Ym-1, Arginasa 1 y FIZZ que corresponden a AAMs fue mayor en el grupo *T.crass*+CAC. En contraparte, INOS la cual es una enzima asociada a procesos inflamatorios fue claramente reducida en el grupo *T. crass*+CAC a diferencia del grupo CAC. Nuestros

datos concuerdan con lo ya reportado pues la citocina IL-4 que es producida en infecciones por helmintos regula negativamente la activación de macrófagos pro-inflamatorios y promueve a los AAMs que como mencionamos anteriormente también tienen la habilidad de reparar el tejido dañado, e incluso esta citocina está implicada en la diferenciación de las células caliciformes [54] las cuales fueron mayormente expresadas tras la infección por *Taenia*. También, en CAC la ruptura de la barrera epitelial genera la translocación de la microbiota a la lámina propia del intestino y la amplificación de la respuesta inflamatoria. La activación de la inflamación por la microbiota es mediada en parte por TLRs, previamente había sido reportada la capacidad de la infección por *T. crassiceps* de bloquear la señalización de TLR en macrófagos, inhibiendo la producción de IL-12, IL-23, TNF-α y ON las cuales son moléculas importantes en el desarrollo de CAC. Para comprobar si la infección por *Taenia* en este modelo *in vivo* podría generar el bloqueo de la señalización mediada por TLR, realizamos cultivos de colon *ex-vivo* y evaluamos su respuesta tras el estímulo con LPS. Nosotros encontramos una menor producción de TNF-α (proteína producida por la señalización de LPS/TLR) tras el estímulo inflamatorio con LPS en los sobrenadantes de los cultivos de colon provenientes del grupo *T. crass*+CAC, a diferencia del grupo CAC. Por lo que nuestros datos sugieren que la infección por *Taenia* regula el microambiente inflamatorio e induce un fenotipo inmunotolerigenico en colon, lo que podría evitar la inflamación exacerbada inhibiendo así la tumorigenesis. Respecto a poblaciones celulares inflamatorias que han sido asociadas a procesos tumorigénicos [55], la infección por *Taenia* regula el reclutamiento de monocitos inflamatorios $CD11b^{+}Ly6C^{hi}CCR2^{+}$ tanto en circulación como en tejido de colon, implicando una fuerte actividad reguladora disparada por este helminto el cual se encontraba localizado en cavidad peritoneal. En conclusión, a través de diversos posibles mecanismos de acción la previa infección extraintestinal con *Taenia crassiceps* regula negativamente el establecimiento del cáncer de colon asociado a colitis.

Artículo 3.

Blanca E. Callejas, Mónica G. Mendoza-Rodríguez, Sandy Reyes- Martínez, C. Ángel Sánchez-Barrera, Miriam Rodríguez-Sosa, Norma L. Buenrostro, Diana Martínez-Saucedo, Yolanda I. Chirino, Sonia A. León-Cabrera, Carlos Pérez-Plasencia, Felipe Vaca-Paniagua, Luis E. Arias-Romero and Luis I. Terrazas. **Helminth-derived molecules inhibit colitis-associated colon cancer development through NF-kB and STAT3 regulation.** 2019. *International Journal of Cancer.* ENVIADO

Debido a nuestro hallazgo anterior donde la infección modifica negativamente el desarrollo de CAC, se consideró esto como una prueba del concepto en el que la inmuroregulación generada por este helminto pudo evitar el desarrollo tumoral en el colon, por lo que decidimos retar más a nuestro sistema a través del uso de los productos TcES. Reportes previos indican que los TcES son capaces de interferir con la maduración de las células dendríticas las cuales adquieren la capacidad de inducir a linfocitos T $CD4^+$ a un fenotipo Th2, y en modelos *in vivo*, la administración de TcEs favorece una fuerte respuesta inmunoreguladora. Recientemente, se ha demostrado que el uso de infecciones por helmintos en pacientes con enfermedades infamatorias no ha sido tan exitoso como se esperaba (ClinicalTrials.gov Identifier: NCT01433471, NCT01576471, NCT01953354) como había sido demostrado en diferentes modelos animales. Especialmente en las IBD, UC y CD se observó que algunas de las infecciones utilizadas generaron reacciones colaterales dañinas a los pacientes [56]. Por lo que los estudios más recientes se han enfocado a utilizar derivados de parásitos que generen una protección similar a la de la infección pero sin el potencial dañino del parásito completo. En nuestro laboratorio nuestro grupo de trabajo ha estudiado el efecto de los TcES (glicoproteínas mayores de 50 kDa) y su capacidad de revertir el avance de algunas enfermedades inflamatorias o autoinmunes como la encefalomelitis autoinmune experimental (EAE), diabetes tipo 1 y colitis experimental aguda [46,47,51]. Otros autores han estudiado también el efecto de algunos derivados de helmintos tales como *Echinococcus, Brugia, Ascaris, Caenorhabditis* e *Hymenolepis* en modelos

similares asociados a inflamación [44]. Sin embargo, existen limitados estudios de como las moléculas derivadas de este tipo de parásitos pueden evitar o alterar el desarrollo de diferentes clases de cáncer (para detalles favor de leer el artículo de revisión "*Parasites as negative regulators of cancer*" anexo a esta tesis).

El objetivo de este estudio fue determinar el efecto de los productos excretados/secretados del helminto *Taenia crassiceps* (TcES) y sus posibles mecanismos anti-tumorales en un modelo murino de cáncer de colon asociado a colitis (CAC). La idea se enfocó en la generación del daño en el hospedero a través de la inducción de CAC y posterior administración de un tratamiento a base de TcES. Así los TcES fueron administrados en el día 26 después de la inducción de CAC vía i.p. tres veces por semana a una dosis de 200 ug por ratón, tratamiento que se mantuvo hasta el día 68.

Nuestros datos indican que después de inducir el CAC, la administración de TcES disminuyó significativamente el número y el tamaño de los tumores colónicos, y alrededor del 45% de los ratones estuvo libre de tumores. La patología del CAC evaluada a través del infiltrado celular y mantenimieno de las células caliciformes en colon fue claramente limitada tras la administración de TcES. En el microambiente del colon, el tratamiento con TcES redujo las citocinas inflamatorias asociadas a procesos oncogénicos tales como IL-1β, TNF-α, IL-33 e IL-17, así como la migración de neutrófilos a la barrera epitelial del intestino y menor daño al DNA determinado a través de la expresión de 8-hidroxiguanosina. Con el objetivo de identificar el posible mecanismo de acción antitumoral de los TcES decidimos evaluar diversas vías de señalización implicadas en la tumorigenesis en colon.

Durante el desarrollo del CAC la desregulación de la vía de señalización de STAT3 le confiere a las células epiteliales la capacidad de proliferar descontroladamente. Nosotros encontramos que la administración de TcES reguló negativamente la vía de señalización de STAT3 a pesar de existir grandes cantidades de IL-6 (potente activador de STAT3) en sobrenadantes de cultivos de colon, también encontramos una disminución substancial de la transcripción del IL-6R lo que podría sugerirnos una señalización dañada de IL-6/STAT3 en

ausencia de IL-6R, esto fue comprobado a través de la disminución de la expresión de ciclina D1, una proteína inducida por la activación de STAT3. Por otro lado, la DNMT1 que es mayormente expresada en células displásicas y que es inducida por IL-6 independiente de STAT3 también fue evaluada, nuestros resultados mostraron que la ausencia del receptor de la IL-6 también daña la expresión de DNMT1 en el colon del grupo CAC+TcES.

Debido a que diversas vías de señalización como Wnt/β-catenina son activadas por la señalización mediada por AKT que como se mencionó en la sección de introducción puede ser activada por IL-6, y que previamente encontramos que los TcES inhiben la transcripción de IL-6R, decidimos evaluar si la activación de AKT podría verse afectada por los TcES en el modelo de CAC. Encontramos una clara disminución de la fosforilación de AKT en el residuo de la serina 473 en el grupo CAC+TcES a diferencia del grupo CAC, por lo que evaluamos a PTEN, uno de los reguladores río arriba de la activación de Akt. Sin embargo, no observamos diferencias en la expresión de esta fosfatasa respecto al grupo control.

Una vez activada AKT una de sus varias funciones es la fosforilación de GSK3β, lo que conlleva a la acumulación de la β-catenina nuclear e interacción con el complejo TCF/LEF para inducir la transcripción de genes asociados a proliferación celular, en el grupo CAC+TcES no observamos grandes cantidades de β-catenina nuclear a diferencia del grupo CAC, por lo que los TcES generaban la inhibición de la desregulación de AKT y β-catenina lo cual fue comprobado a través de la medición de c-Myc el cual fue expresado exclusivamente en las IEC´s transformadas del grupo CAC y totalmente ausente en el grupo CAC+TcES.

Por otro lado, NF-κB es el principal regulador maestro en procesos inflamatorios y es asociado a procesos oncogénicos como proliferación y evasión de la apoptosis. Debido a que los TcES regulan el microambiente tumoral decidimos evaluar la activación de NF-κB en el modelo de CAC. Encontramos que la administración de TcES inactiva la vía de NF-κB, reduce la transcripción de TLR4 y la expresión de BCL-2 (proteína antiapoptótica) en el colon de animales tratados con TcES, mientras que BCL-2 es altamente expresada en las células epiteliales transformadas.

Para determinar el papel directo y el posible afecto antitumoral de TcES sobre las células epiteliales decidimos evaluar el efecto del estímulo de los TcES sobre la línea celular de cáncer de colon de humano RKO. Encontramos que el estímulo de 12.5 µg/ml de TcES genera la disminución en la proliferación celular de ambas líneas de cáncer de colon sin ningún efecto citotóxico. De manera independiente evaluamos si los TcES podrían generar cambios en la morfología de las células de cáncer de colon, encontramos que en los cultivos en 3D el estímulo de TcES promovió la reorganización del citoesqueleto de actina en ambas líneas celulares, alterando la morfología celular y formando colonoesferas, estructuras características asociadas con un bajo grado de agresividad. En conclusión, en la presente investigación demostramos un efecto notable de las moléculas derivadas de un helminto en la supresión del cáncer colorrectal, a través de la inactivación de vías de señalización proinflamatorias y protumorigénicas.

Artículo de revisión

Callejas BE, Martínez-Saucedo D, Terrazas LI. **Parasites as negative regulators of cancer.** 2018. *Biosci Rep.* 22;38(5). doi: 10.1042/BSR20180935

En los últimos años se ha reportado que diferentes tipos de infecciones causadas por bacterias, helmintos y virus pueden inducir o promover diferentes tipos de cáncer. Actualmente, más del 20% de los cáncer se han relacionado a diferentes agentes infecciosos como el virus del papiloma humano (VPH), virus de Epstein-Barr, Hepatitis C, *Helicobacter pylori, Schistosoma haematobium, Opisthorchis viverrini y Clonorquis sinensis* [36]. Sus mecanismos oncogénicos son variados, entre los que se encuentran: la inducción de expresión de oncogenes, producción de moléculas genotóxicas e inflamación caracterizada por el aumento en la generación de especies reactivas de oxígeno (ROS) causantes de daño al ADN, y activación de vías de señalización que promueven la proliferación y evasión de la apoptosis de las células ya transformadas. Sin embargo, en años recientes un enfoque diferente ha sido implementado en el impacto antitumoral de las enfermedades parasitarias causadas por algunos protozoos y helmintos, ya que tales infecciones pueden afectar varios "hallmark" del cáncer. Protozoarios tales como *Toxoplasma gondii* y *Trypanosoma cruzi* tienen un efecto antitumoral sobre melanoma, fibrosarcoma, cáncer de pulmón, cáncer de mama y cáncer de colon a través de la capacidad anti-angiogénica (inducción de hipoxia), la reactivación de la respuesta inmune (Inducción de la respuesta inmune humoral y Th1) y la inducción de la apoptosis (a través de caspasas) [57–64]. Por otro lado, *Taenia crassiceps* es capaz de regular la respuesta inflamatoria inductora de cáncer, ya que la infección previa con éste cestodo inhibe la tumorigenesis en colon[65]. *Echinococcus granulosus* tiene diferentes mecanismos antitumorales, como la reactivación de la respuesta inmune y el efecto antiproliferativo (a través del arresto celular) en un modelo de fibrosarcoma, cáncer de mama y colon, así como la *Trichinella spiralis* con un efecto regulador de la invasión y la metástasis (a través de la regulación de quimiocinas) y las señales antiproliferativas en melanoma y en las líneas celulares de hepatoma de humano y la línea celular de leucemia mieloide crónica de humano [66–68]. A pesar de que éstos parásitos han sido reportados por su efecto como

moduladores en la progresión de cáncer, los mecanismos específicos de acción aún no son conocidos. En conclusión, debido al claro efecto regulador de diversos parásitos protozoarios y helmintos sobre algunas neoplasias, es necesario considerar el estudio detallado de su acción antitumoral, lo cual podría conducir al descubrimiento y uso de nuevas moléculas a partir de estos agentes biológicos y ser usadas como terapia adyuvante en el tratamiento de varios tipos de cáncer.

DISCUSIÓN GENERAL

Actualmente, la inflamación es ampliamente considerada como uno de los "hallmark" del cáncer. La inflamación está relacionada con diferentes etapas de la tumorigénesis, que incluyen; iniciación, promoción, y progresión del cáncer, así como en la resistencia a terapias antitumorales [69]. Diversos reportes sugieren la capacidad de varias infecciones parasitarias para modular el desarrollo del cáncer, en muchos casos favoreciendo la progresión del tumor y en otros, sorprendentemente, inhibiendo la tumorigénesis [36,40].

En nuestro grupo de investigación reportamos que la infección previa con *Taenia crassiceps* redujo la expresión de citocinas inflamatorias, indujo el reclutamiento de AAMs en colon mientras redujo la patología asociada a la colitis ulcerativa [65]. Debido a que la colitis representa un factor de riesgo del desarrollo del cáncer colorrectal, evaluamos el papel de la infección por *Taenia crassiceps* y el efecto de sus productos excretados/secretados en un modelo de cáncer de colon asociado a colitis.

En la primera parte del proyecto de investigación "Efecto anti-inflamatorio de *Taenia crassiceps* durante el desarrollo de CAC", observamos que la previa infección extraintestinal de *T. crassiceps* en CAC regula la producción de citocinas inflamatorias, el reclutamiento de neutrófilos, así como los monocitos asociados a procesos inflamatorios, tanto en circulación como en tejido de colon. Por otro lado, debido a que infecciones crónicas por helmintos inducen macrófagos activados alternativamente, determinamos F4/80 y por separado los marcadores Ym-1, FIZZ y Arg-1 en colon, como esperábamos, estos fueron sobreexpresados en ratones del grupo previamente infectado con *T. crassiceps* y posterior inducción de CAC. Es ampliamente reportado el papel de los macrófagos asociados a tumor (TAMs) como promotores de diversas neoplasias los cuales poseen características similares a los AAMs [70]. Sin embargo, la presencia de estos marcadores y el amplio infiltrado de macrófagos en colon no promovieron la carcinogénesis. Esto podría deberse a que la inflamación es un evento primario al desarrollo de cáncer colorrectal, por lo que la infección previa con *T. crassiceps* podría evitar la respuesta inflamatoria exacerbada controlando el desarrollo de este tipo de cáncer. La presencia temprana de AAMs en colon podría sugerir la reparación del tejido en condiciones adversas sin promover la tumorigénesis. Concluimos

que la previa infección extraintestinal con *T. crassiceps* inhibe el desarrollo de cáncer de colon asociado a colitis.

Sin embargo, el uso de agentes biológicos vivos como terapia profiláctica en enfermedades asociadas a procesos inflamatorios es una idea poco viable debido a los potenciales efectos adversos que podría generar tener un agente patogénico vivo por semanas en el hospedero o paciente, por lo que decidimos evaluar el efecto de las moléculas derivadas de *T. crassiceps* una vez iniciado el CAC. En la segunda parte del proyecto, demostramos el potente efecto antiinflamatorio y antitumoral de los productos excretados/secretados de *T. crassiceps* sobre la activación de NF-κB y STAT3 en el desarrollo del cáncer de colon asociado a inflamación. Así como el efecto regulador de los productos derivados de *T. crassiceps* sobre la proliferación celular y formación de colonosferas en la la línea celular de cáncer de colon humano RKO.

Los principales cambios patológicos observados en el desarrollo del cáncer son promovidos por el microambiente inflamatorio [11]. La citocina protumoral IL-6 en cáncer colorectal es producida principalmente por macrófagos de la lámina propia y células dendríticas, y a menudo se correlaciona con un pobre pronóstico [71]. El efecto proliferativo y de supervivencia de IL-6 es mediada a través de la activación del factor de transcripción oncogénico STAT3, y la vía de señalización PI3K-AKT a través de la señalización de IL-6R y su receptor común GP130, conduciendo a la transcripción de genes blanco incluyendo la ciclina D1, la cual es una proteína reguladora del ciclo celular [25]. Nuestros resultados claramente muestran que TcES inhibió la activación de STAT3 a pesar de haber grandes cantidades de IL-6 en el tejido de colon. Dado que resultados previos indican que el estímulo por los productos TcES sobre macrófagos pueden inducir altos niveles de las proteínas reguladoras negativas de STAT1, es decir SHP1, SOCS1 y SOCS3 [45], nosotros evaluamos diferentes reguladores negativos de STAT3. Sin embargo, ni el incremento de SOCS3 ni PIAS3 por la administración de TcES fue substancial para la regulación negativa de esta vía. Como mencionamos anteriormente, el complejo IL-6/IL-6R/GP130 juega un papel decisivo para la activación de STAT3 [72 21,73]. Encontramos que la disminución de la fosforilación de STAT3 es mediada por una menor transcripción del IL-6R, por lo que su ausencia impide el consumo

de la IL-6 en el microambiente tumoral inhibiendo la activación de STAT3. En CAC y cáncer de piel, la ablación de STAT3 en IEC reduce el crecimiento tumoral [22,23] y el bloqueo de la señalización IL-6/IL-6R suprime la angiogénesis y el crecimiento tumoral de carcinoma de células escamosas orales de humano y de mama [74]. En línea con lo anterior, nuestros datos sugieren un posible mecanismo antitumoral de TcES a través del bloqueo de la señalización de STAT3 inhibiendo la proliferación de las IEC´s en el modelo de CAC.

Retomando la causa de las grandes cantidades de IL-6 en tejido de colon, en infecciones por helmintos la IL-6 es una citocina predominante, su capacidad pleiotrópica promueve una respuesta tipo Th2 así como la polarización de macrófagos a AAMs, a diferencia de su papel en cáncer, en donde la IL-6 junto con TGFβ inducen a la células T naive a un fenotipo Th17. Por lo que la IL-6 podría tener un papel regulador de la respuesta inflamatoria en el modelo de CAC tras el estímulo por TcES a diferencia del grupo CAC, en donde su actividad es protumoroigénica. La disminución de la transcripción de IL-6R respecto al grupo CAC, podría deberse a las vías activadas por el estímulo de IL-6 en microambientes diferentes, a una posible autoregulación de la señalización mediada por IL-6, así como a la proporción de las células epiteliales que necesitan de la señalación de STAT3 a través del complejo de IL-6/IL-6R para su proliferación. Sin embargo, más datos deben ser generados para esclarecer el posible mecanismo de regulación de la transcripción de IL-6R por el estímulo con TcES.

Además de STAT3, la IL-6 puede activar la señalización mediada por AKT la cual está implicada en procesos de proliferación celular en el desarrollo de CAC. Observamos que la ausencia de la fosforilación de AKT en el grupo CAC+TcES fue un evento independiente de la expresión del regulador negativo PTEN ya que no encontramos diferencias entre el grupo control y CAC+TcES. Rio abajo, la fosforilación de AKT facilita la acumulación de β-catenina en el núcleo para interactuar con el factor de transcripción TCF/LEF promoviendo la proliferación celular además de procesos de invasividad [10,75]. Como los resultados claramente muestran, TcES inhibe la acumulación de β-catenina en el núcleo, así como la expresión de la proteína oncogénica c-Myc en el modelo de CAC. No es claro si la reducción de la fosforilación de AKT y por lo tanto la disminución de la β-catenina nuclear es un evento

colateral de la disminución de la expresión de IL-6R en el grupo CAC+TcES, o si otras citocinas como TNF-α e IL-1β (también activadoras de la señalización mediada por AKT) las cuales fueron claramente reducidas por los TcES en CAC, evitan la activación de AKT.

En el microambiente inflamatorio además de contener citocinas inflamatorias que activan diversas vías de señalización potenciando la proliferación de las células, la presencia de células inflamatorias como lo neutrófilos es característica. Los neutrófilos, sirven como fuente principal de especies de oxígeno reactivas (ROS) que son capaces de inducir daño al DNA e inestabilidad genética [11]. El receptor de quimiocina CXCR2 es localizado en neutrófilos y su ligando CXCL2 es expresado en IEC´s lo que promueve su reclutamiento al colon [76].El tratamiento con TcES no modifica los niveles de CXCL2 por lo que el porcentaje de neutrófilos (Ly6G) infiltrados al colon no difiere al grupo CAC. Interesantemente, la localización de los neutrófilos es diferente por el estímulo de TcES, ya que claramente los neutrófilos son identificados en la base de la lámina propia a diferencia del grupo CAC, en donde la infiltración de neutrófilos es observada cercana a la barrera epitelial. La molécula 1 de adhesión intercelular (ICAM1) es altamente expresada en la zona apical de las IEC´s bajo condiciones inflamatorias, su expresión media la migración transepitelial (TEM) y el arresto de los neutrófilos en el colon [77,78]. Estos datos son consistentes con nuestros resultados, ya que TcES disminuye la producción de ICAM1 sugiriendo la inhibición de la migración de los neutrófilos a la barrera epitelial del colon. Otro rasgo interesante, es que el daño al DNA generado por ROS fue menor por los TcES en el modelo de CAC aun a pesar de haber presencia de células Ly6G positivas en colon. Esto podría sugerir que los TcES inducen una población de neutrófilos con menor capacidad de producir daño al DNA mediada por ROS.

Diferentes estudios han demostrado que el bloqueo de IL-1β, TNF-α o el uso de ratones deficientes de IL-17 atenúan notablemente la inflamación colónica lo que disminuye el establecimiento de tumores de colon [79,80], por lo que estas citocinas son promotores clave de la inflamación local. Además de este microambiente inflamatorio constante y a una clara disfunción de la barrera epitelial, las IEC´s están expuestas a una gran variedad de microorganismos y sus productos, tales como como LPS, durante el desarrollo de CAC, lo

que resulta en la activación prolongada de la señalización mediada por NF-κB, favoreciendo aún más la inflamación local [11]. Como mencionamos anteriormente, la exposición constante a TcES disminuyó la producción local de IL-1β, TNF-α, IL-23 e IL-17, todas ellas citocinas inflamatorias con capacidad oncogénica [8]. Además de citocinas clásicamente asociadas con inflamación y tumorigénesis de colon, los niveles de otras dos citocinas no convencionales, IL-31 e IL-33, parecen estar moduladas por TcES. La producción de IL-31, una citocina recientemente asociada con la reparación del tejido intestinal[81], fue reducida significativamente en ratones que albergan tumores, mientras que los ratones tratados con TcES mantuvieron la producción normal de esta citocina, lo que sugiere que TcES puede mejorar la reparación del tejido después del daño inducido por AOM/DSS. En claro contraste, la producción de IL-33 se redujo con el tratamiento con TcES durante CAC. IL-33 es considerada una alarmina y es liberada por las células epiteliales después de daño o necrosis [82]. Por lo que, TcES podría prevenir el daño tisular y favorecer la rápida reparación del tejido. Además, la IL-33 induce la expresión de ICAM-1 a través de la activación de NF-kB [83], que a su vez favorece la inflamación y la progresión del cáncer. Por lo tanto, la regulación por disminución de IL-33 mediada por TcES en el colon puede desencadenar una disminución en la expresión de ICAM1.

La activación constante de NF-κB en las células del sistema inmunológico aumenta y mantiene las respuestas inflamatorias que promueven el cáncer. Sin embargo, en las células cancerosas, la activación de NF-κB estimula la proliferación, supervivencia, invasión, transición epitelial-mesénquima, angiogénesis y metástasis [14,84]. Aquí encontramos que durante el desarrollo de CAC los TcES inhibieron la activación de NF-κB en el tejido del colon, posiblemente a través de la disminución de la expresión de TLR4, también observamos disminución en la expresión de la proteína antiapoptotica BCL-2. Estos hallazgos pueden explicar parcialmente el mecanismo por el cual los TcES redujeron la carga tumoral en CAC. Para investigar más a fondo la capacidad de TcES para reducir la tumorigénesis en colon en las etapas iniciales, extendimos el análisis a una línea celular de cáncer de colon humano completamente transformada (RKO)

Encontramos que en cultivos 3D, TcES promovió la reorganización de citoesqueleto de actina de RKO, alterando la morfología celular y formando colonoesferas. Como se informó anteriormente en otros modelos de cáncer, la reorganización de las células tumorales se asocia con un bajo grado de agresividad; Por lo tanto, nuestros datos sugieren un efecto regulador de TcES en las señales asociadas con la migración y la invasividad.

En general, nuestro estudio actual es el primero en demostrar que las moléculas derivadas de helmintos regulan negativamente la transducción de las señales proinflamatorias y protumoraless mediante la supresión de la señalización de STAT3 y NF-κB, previniendo la tumorigénesis del colon. Considerando los hallazgos obtenidos, proponemos un modelo hipotético que ilustra el mecanismo mediante el cual TcES puede bloquear varias vías en el desarrollo de CAC (Fig 6. Artículo 3).

Es importante destacar que los efectos de TcES han sido observados también en células de humano, ya que TcES evita la maduración de las DC inflamatorias de humano en respuesta a LPS y la activación de STAT1 en macrófagos de humano en respuesta a IFN-γ [45,48,87]. Nuestros datos muestran que TcES previno la activación de NF-κB en respuesta a LPS en dos diferentes líneas celulares epiteliales de colon humano transformadas. Por lo tanto, los TcES son probablemente una terapia adyuvante antitumoral prometedora para la carcinogénesis asociada a la inflamación.

En conclusión, la infección por *Taenia crassiceps* así como sus antígenos de excresión/secresión reducen el desarrollo del CAC a través de la modulación de la inflamación y señales protumorigénicas.

CONCLUSIONES GENERALES

- La infección por *Taenia crassiceps* induce AAMs y regula la colitis aguda experimental.
- La infección previa por *Taenia crassiceps* inhibe el desarrollo del CAC.
- Moléculas derivadas de *Taenia crassiceps* (TcES) inhiben el desarrollo del CAC.
- TcES modula el microambiente inflamatorio protumoral en el modelo de CAC al inhibir la producción de IL-23, ICAM1, IL-17 y TNF-α, y modula el reclutamiento de neutrófilos a la barrera epitelial de colon.
- TcES regula la transcripción del receptor de IL-6 y TLR4 en el desarrollo de CAC.
- La administración de TcES inhibe la activación de AKT y la acumulación nuclear de β-catenina disminuyendo la expresión de c-Myc.
- TcES inhibe la activación NF-kB y STAT3 disminuyendo la expresión de BCL-2 y ciclina D1 en CAC.
- TcES regula la proliferación, sin inducir apoptosis, de una línea celular derivada de cáncer de colon humano.
- TcES induce la formación de colonosferas de la línea celular RKO.

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Apéndice I

Artículo de investigación 1: Ledesma-Soto Y, Callejas BE, Terrazas CA, Reyes JL, Espinoza-Jiménez A, González MI, León-Cabrera S, Morales R, Olguín JE, Saavedra R, Oghumu S, Satoskar AR, Terrazas LI. Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins. 2015. *Biomed Res Int.* 2015;2015:563425. doi: 10.1155/2015/563425

Artículo de investigación 2: León-Cabrera S, Callejas BE, Ledesma-Soto Y, Coronel J, Pérez-Plasencia C, Gutiérrez-Cirlos EB, Ávila-Moreno F, Rodríguez-Sosa M, Hernández-Pando R, Marquina-Castillo B, Chirino YI, Terrazas LI. Extraintestinal Helminth Infection Reduces the Development of Colitis-Associated Tumorigenesis. 2014. *Int J Biol Sci.* 10(9):948-56. doi: 10.7150/ijbs.9033.

Artículo de investigación 3: Blanca E. Callejas, Mónica G. Mendoza-Rodríguez, Olga Villamar-Cruz, Sandy Reyes- Martínez, C. Angel Sánchez-Barrera, Miriam Rodríguez-Sosa, Norma L. Buenrostro, Diana Martínez-Saucedo, Yolanda I. Chirino, Sonia A. León-Cabrera, Carlos Pérez-Plasencia, Felipe Vaca-Paniagua, Luis E. Arias-Romero and Luis I. Terrazas. Helminth-derived molecules inhibit colitis-associated colon cancer development through NF-kB and STAT3 regulation. 2019. *International Journal of cancer.* ENVIADO

Artículo de revisión: Callejas BE, Martínez-Saucedo D, Terrazas LI. Parasites as negative regulators of cancer. 2018. *Biosci Rep.* 22;38(5). doi: 10.1042/BSR20180935

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Research Article

Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins

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Chronic inflammation of the intestinal mucosa is characteristic of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Helminth parasites have developed immunomodulatory strategies that may impact the outcome of several inflammatory diseases. Therefore, we investigated whether *Taenia crassiceps* infection is able to decrease the inflammatory effects of dextran sulfate sodium- (DSS-) induced ulcerative colitis in BALB/c and C57BL/6 mice. Preinfection significantly reduced the manifestations of DSS-induced colitis, as weight loss and shortened colon length, and decreased the disease activity index independently of the genetic background of the mice. *Taenia* infection decreased systemic levels of proinflammatory cytokines while increasing levels of IL-4 and IL-10, and the inflammatory infiltrate into the colon was also markedly reduced. RT-PCR assays from colon showed that *T. crassiceps*-infected mice displayed increased expression of Arginase-1 but decreased expression of iNOS compared to DSS-treated uninfected mice. The percentages of T regulatory cells were not increased. The adoptive transfer of alternatively activated macrophages (AAMΦs) from infected mice into mice with DSS-induced colitis reduced the severity of colon inflammation. Administration of indomethacin abrogated the anticolitic effect of *Taenia*. Thus, *T. crassiceps* infection limits the pathology of ulcerative colitis by suppressing inflammatory responses mechanistically associated with AAMΦs and prostaglandins.

1. Introduction

Helminth parasites have developed complex strategies to modulate the immune responses of their hosts through utilizing versatile immunoregulatory mechanisms to avoid immune effector cells and molecules. These parasites bias the immune response toward Th2 and/or a regulatory environment associated with high levels of IL-4, IL-13, IL-9, IL-5, and IL-10; also, infection with helminths compromises immunity to other unrelated infections and may also affect the efficacy of vaccines [1]. Various cell populations are affected by helminth infections, including macrophages, dendritic cells (DCs), T regulatory cells (Treg), mast cells, and neutrophils [2]. Thus, helminths use multiple means to escape or modulate the immune response in their hosts [3]. A growing body of evidence in recent years has shown

that immunomodulatory activities displayed by helminths can impact in different ways the outcomes of several inflammatory diseases, including multiple sclerosis (MS), arthritis, type 1 diabetes (T1D), and inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease [4].

One inflammatory disease of alarming frequency is IBD. Although these diseases were considered rare 50 years ago, today some developed countries report 1 patient for every 250 people [5]. In Europe up to 0.3% of the population suffer from IBD [6]. An association has been suggested between an absence of helminth infection and the increase in cases of IBD [4]. Rodent models of IBD have been used to study the mechanisms underlying the development of these inflammatory diseases [7], and several experimental studies support the idea that IBD (mainly ulcerative colitis) can be regulated by helminth infection [8, 9]. Human trials have demonstrated some efficacy for patients but with undesirable side effects [10–12]. Nevertheless, seven different species of helminths, mainly gastrointestinal ones, have been tested for their role in modulating the development of ulcerative colitis, some with adverse effects [10, 11, 13–15].

Taenia crassiceps (class *Cestoda*) is a helminth parasite that can be found in its adult form in the small intestine of canids and in its larval stage (metacestode) in the muscles and peritoneal and pleural cavities of rodents. An interesting feature of *T. crassiceps* is its ability to reproduce asexually through budding at the larval stage. This characteristic permits the parasite to remain in and colonize its hosts for long periods of time; thus, 6–8 weeks after the intraperitoneal (i.p.) inoculation of 10–20 metacestodes, hosts can harbor hundreds of parasites. In addition, the parasite in its larval stage is innocuous to humans, is macroscopic in size, does not kill the host, and is able to cause chronic infection with a minimum amount of damage in mice. We found an inhibition of proinflammatory responses, induction of Th2-biased immune responses, myeloid-derived suppressor cells, impairment of DC maturation, and lymphocyte proliferative responses, as well as recruitment of alternatively activated macrophages (AAMΦs) during *T. crassiceps* infection, reviewed in [16]. Such immunoregulatory properties of this helminth had important beneficial effects on the development of experimental autoimmune encephalomyelitis (EAE, a murine model for multiple sclerosis) and T1D without side effects on the hosts [17, 18], whereas no effect at all was observed on arthritis [19].

Most of the effects of helminths on colitis have been studied using gastrointestinal infections, and consequently the impact of infection with helminths on organs outside the parasite's location has received much less attention [20]. For this reason and given the anti-inflammatory and immunoregulatory mechanisms of *T. crassiceps* infection, in this work we evaluated the effect of an extraintestinal infection on the development of dextran sulfate sodium- (DSS-) induced colitis.

2. Material and Methods

2.1. Mice. Female BALB/c or C57BL/6 mice 6–8 weeks of age were purchased from Harlan Laboratories (México) for use in some experiments. Mice were maintained in a pathogen-free environment at the FES-Iztacala, UNAM, animal facility according to Faculty Animal Care and Use Committee and government guidelines (official Mexican regulation NOM-062-ZOO-1999), which are in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). Mice were sacrificed using a CO_2 chamber, and all efforts were made to minimize pain. In some experiments C.Cg-*Foxp3*^{tm1Tch}/J reporter mice (Jackson Labs, USA) were used to detect the presence of T regulatory cells.

2.2. Parasites and Infection. Metacestodes of *T. crassiceps* were harvested from the peritoneal cavities of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS; 0.15 M, pH 7.2). Experimental infection was achieved via i.p. injection of 20 small (approximately 2 mm in diameter) nonbudding cysticerci of *T. crassiceps* suspended in 0.3 mL PBS per mouse (BALB/c). Because C57BL/6 mice are resistant to low doses (10–20 metacestodes) of *T. crassiceps*, we infected all C57BL/6 mice with 40 metacestodes.

2.3. Development and Assessment of DSS-Induced Colitis. DSS (MW: 35,000–50,000; MP Biomedicals, Solon, OH, USA) was administered ad libitum dissolved at 4% in drinking water for 7 to 10 days.

2.4. Assessment of Disease Activity Index (DAI) Score and Weight Loss. To assess the severity of colitis, we monitored DAI scores and weight loss daily. The DAI score was calculated as the sum of the diarrheal score and the bloody stool score as follows: 0 = normal stool and normal-colored stool, 1 = mildly soft stool and brown stool, 2 = very soft stool and reddish stool, and 3 = watery stool and bloody stool.

2.5. Histology. Colon tissue samples were fixed in 10% formalin and embedded in paraffin. Then 5 micrometer-thick tissue sections were prepared and stained with hematoxylin and eosin (HE) to evaluate mucosal damage. The sections were also stained with Alcian blue to evaluate the presence of goblet cells. We calculated the number of Alcian blue-positive goblet cells per five power fields (40x) using an Axio Vert.A1 microscopy (Carl Zeiss, Gottingen, Germany).

2.6. Cytokine Measurement. IL-4, IL-10, IL-17A, IL-17E, and TNF-α levels were quantified in mouse serum at the indicated times. Kits were used according to the manufacturer's instructions (Peprotech México, Mexico City, Mexico, and Biolegend, San Diego, CA, USA, for IL-17E).

2.7. Peritoneal Macrophage Purification, Adoptive Transfer, and In Vitro Suppression. Peritoneal exudate cells were isolated from the peritoneal cavities of *T. crassiceps*-infected mice 8 weeks after infection. Fc receptors were blocked by incubating the cells with mouse serum for 10 min at 4°C. Then cells were labeled with anti-F4/80 APC and anti-mannose receptor (FITC; 0.25 ug/10^6 cells; Biolegend) for 20 min

at 4°C. F480$^+$MR$^+$ and F480$^+$MR$^-$ populations were high-speed-sorted using a FACs Aria III flow cytometer. The viability of the cells was 90%, and purity was 95%. One million purified F4/80$^+$MR$^+$ or F4/80$^+$MR$^-$ cells were injected intraperitoneally into BALB/c mice 2 days after the start of DSS treatment. In addition, the antiproliferative properties of sorted F4/80$^+$MR$^+$ or F4/80$^+$MR$^-$ cells on T cells were tested. Briefly, total CFSE-labeled splenocytes from naïve mice were plated in 96-well plates previously coated with anti-CD3/CD28 (2 μg/mL). After 4 h, F4/80$^+$MR$^+$ or F4/80$^+$MR$^-$ cells were added in different ratios. Proliferation was evaluated after 72 h on CD8- or CD4-gated populations by CFSE dilution assay using a FACSCalibur cytometer.

2.8. Flow Cytometry Analysis of Monocytes and T Regulatory Cells. Single-cell suspensions of circulation and lamina propria obtained during the sacrifice were stained with specific antibodies against CD11b (M1/70), Ly6C (HK1.4), Ly6G (1A8; all from Biolegend), and CCR2 (R&D Systems, USA) for 30 min at room temperature. To isolate colonic lamina propria cells, we flushed colons of their luminal content with cold PBS, opened them longitudinally, and cut them into 0.5 cm pieces. Epithelial cells and mucus were removed via 30 min incubation with HBSS containing 2% FBS, 2 mM EDTA, at 37°C and shaking at 50 g. Colon pieces were digested in DMEM containing 2 mg/mL Collagenase VIII (Sigma) and 40 μg/mL DNase I (Invitrogen) for 2 h at 37°C with shaking at 250 rpm. The digested cell suspension was then washed with DMEM with 10% FBS, passed sequentially through 100 and 40 μm cell strainers, and pelleted by centrifugation at 448 g for 10 min. Cells were subsequently separated by centrifugation through Percoll. Finally, cells from the same groups were pooled for analysis. Analyses of cells were performed using the FACSCalibur system and Cell Quest software (Becton Dickinson).

For the analysis of T regulatory cells, single spleen cells and PECs suspensions were obtained from C.Cg-*Foxp3*^{tm1Tch}/J reporter mice and stained with CD4 and CD25 (Biolegend) and gated on the CD4$^+$ cell population. From this population CD25 and FOXP3 expression were analyzed.

2.9. In Vivo Suppression of Prostaglandin E_2. To block the production of prostaglandin E_2 in mice, we daily subjected mice to i.p. injection with indomethacin (3 mg per kg body weight). Controls were injected with DMSO 0.5% in bicarbonate buffer 5% as a vehicle control.

2.10. Statistical Analysis. Data were analyzed either by one-way analysis of variance followed by Tukey's multiple comparisons test or by unpaired two-tailed t-tests with GraphPad Prism 5 (San Diego, CA, USA).

3. Results

3.1. Taenia crassiceps Infection Decreases the Severity of DSS-Induced Colitis Independent of Genetic Background. We found that i.p. injection with the cestode *T. crassiceps* induces immunomodulation in its hosts. To formally assess the possible role of this extraintestinal helminth infection in the modulation of inflammatory responses in the colon, we explored whether the presence of this parasite in the peritoneal cavity of the host would modulate the severity of disease in an experimental model of ulcerative colitis. BALB/c and C57BL/6 mice previously infected (6 weeks) or not with *T. crassiceps* were exposed to DSS 4% or 3%, respectively, in drinking water for 7–9 days. The DAI was assessed daily as an average of loss of body weight and signs of rectal bleeding and diarrhea. Under such experimental conditions *T. crassiceps*-infected mice of both strains did not lose weight, whereas uninfected mice had significant progressive weight loss over time, weighing up to 20% of their initial body weight less at day 7 of exposure to DSS (Figure 1(a) for BALB/c; data not shown for C57BL/6). In line with these observations, DSS-treated *T. crassiceps*-infected mice developed reduced signs of morbidity (DAI) over the course of the disease compared to uninfected mice similarly treated with DSS (Figure 1(b)). Consistent with this, at necropsy, reduced colon shortening was found in *T. crassiceps*-infected mice after exposure to DSS compared to uninfected mice; this observation was similar for both strains of mice (Figures 1(c)-1(d) for BALB/c and Figure 4(a) for C57BL/6).

We next analyzed the architecture of the colonic structure and evaluated the histopathology associated with DSS-induced colitis. *T. crassiceps*-infected mice displayed less inflammatory infiltrate as assessed via histological slides (Figures 2(a)-2(b) for BALB/c and Figure 4(b) for C57BL/6). Helminth infection also inhibited the development of cryptitis and neutrophil accumulation within epithelial crypts and in the intestinal mucosa, as uninfected mice that received DSS showed large numbers of neutrophils and macrophages in the injured mucosa of the colon, which correlates directly with clinical disease activity and epithelial injury in colitis (Figure 2(a) upper panel and Figure 2(b)). Moreover, *T. crassiceps*-infected mice and those exposed to DSS had normal numbers of goblet cells as assessed by Alcian blue staining compared to DSS-treated uninfected mice (Figure 2(a) lower panel and Figure 2(c)). The diameter of the colonic submucosa was also measured as a sign of tissue damage and was significantly smaller for the infected group exposed to DSS than for the DSS-treated group (data not shown).

3.2. Taenia crassiceps Infection Reduces Levels of Systemic Proinflammatory Cytokines during DSS-Induced Colitis. Several proinflammatory cytokines as well as inflammatory cells are associated with the severity of IBD [21]. Here we explored whether *T. crassiceps*-infected mice exposed to DSS may modulate systemically the expected increase in proinflammatory cytokines. As shown in Figure 3 for BALB/c mice, DSS-induced colitis in uninfected mice generated an increase in circulating levels of TNF-α and IL-17E, two inflammatory cytokines associated with different models of colitis [22]; however, *T. crassiceps*-infected mice displayed lower levels of both cytokines (Figures 3(a)-3(b)). In contrast, infected mice exposed to DSS displayed increased levels of IL-10 and IL-4 compared to uninfected and DSS-treated mice (Figures 3(c)-3(d)). A similar effect was observed in the C57BL/6 strain, in which systemic TNF-α levels were downregulated by the

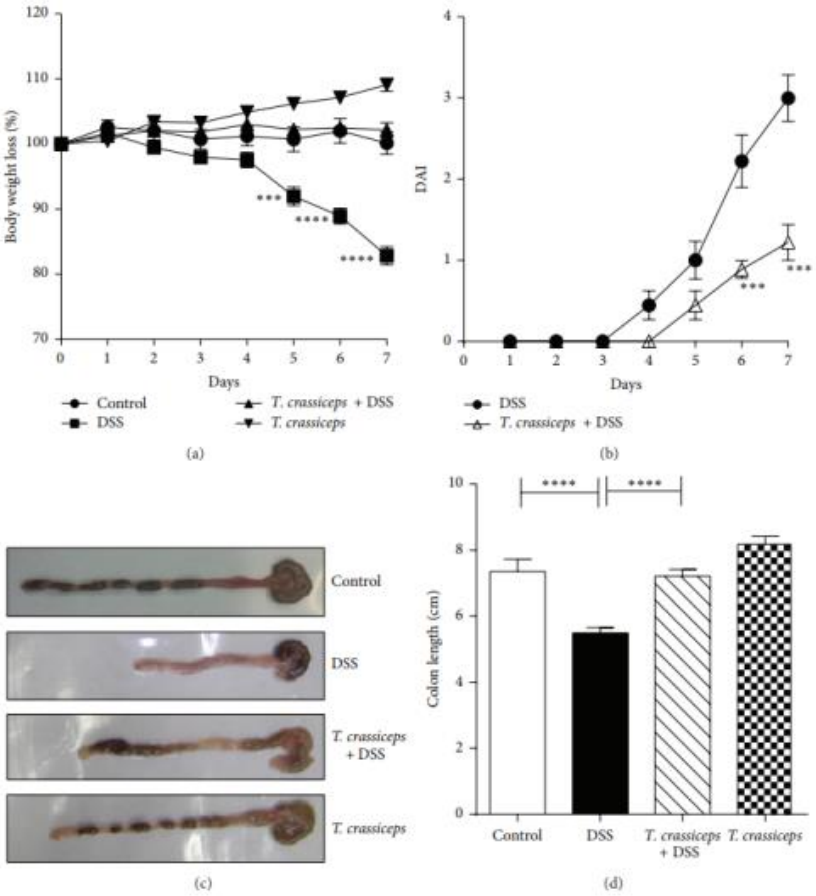

FIGURE 1: *T. crassiceps*-infected mice efficiently control colitis-associated pathology. Course of ulcerative colitis in *T. crassiceps*-infected and uninfected mice following 7 days of treatment with DSS at 4%. (a) Body weight change. (b) Clinical score. (c) Photograph of gross pathology of colons from different groups of mice. (d) Length of colon of infected and uninfected mice with ulcerative colitis. Bars represent the mean ± SD for six mice per group. $^{*}P < 0.05$, $^{***}P < 0.003$. All data are representative of three independent experiments.

presence of *T. crassiceps* infection (Figure 4(c)) but IL-4 and IL-10 levels were significantly elevated (Figures 4(d)–3(e)). Unexpectedly, *T. crassiceps*-infected mice exposed to DSS displayed significant increased levels of IL-17A compared to uninfected and DSS exposed mice (Figure 3(e)).

3.3. Taenia crassiceps Infection during DSS-Induced Colitis Does Not Modify the Population of T Regulatory Cells. Given reports suggesting that the increases in Treg ($CD4^{+}CD25^{+}Foxp3^{+}$) induced by helminth infections are critically involved in the anticolitic effects of these parasites [23–26], we used a Foxp3 reporter mouse to evaluate the percentages of T regulatory cells in the spleen and peritoneal cavity during *T. crassiceps* infection and DSS-induced colitis. As shown in Figure 5, we did not find significant changes in the percentages of T regulatory cells at either location in infected mice or infected and DSS-treated mice compared to DSS-treated uninfected mice.

3.4. Taenia crassiceps Infection Reduces Levels of Systemic and Colonic Inflammatory Monocytes but Increases AAMΦ Markers in Colonic Tissue during DSS-Induced Colitis. To examine changes in the recruitment of inflammatory monocytes to the colon tissue during DSS-induced colitis and the effect of *T. crassiceps* infection in these populations we examined circulating levels of $CD11b^{+}Ly6^{hi}CCR2^{+}$ cells as well as those

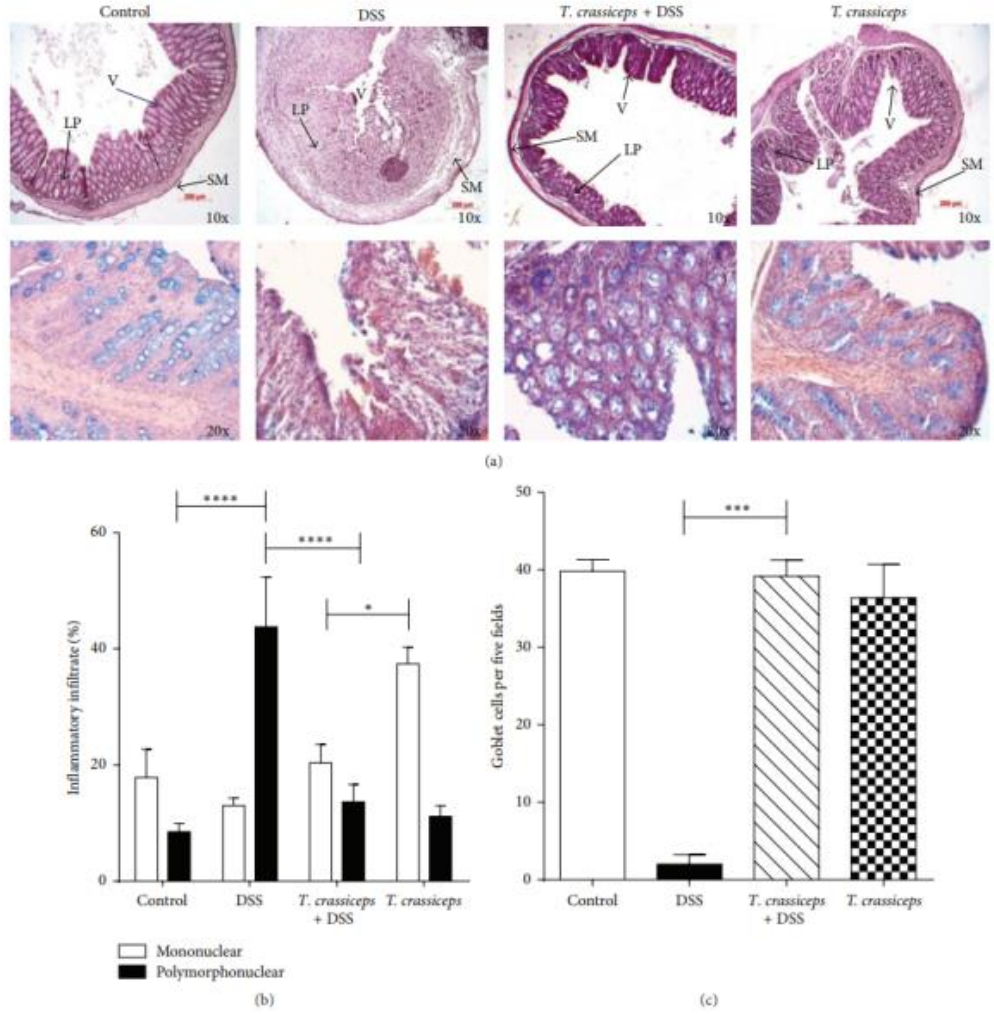

FIGURE 2: *T. crassiceps*-infected mice do not display severe pathology during ulcerative colitis. (a) Upper panel, colon tissue histology stained with H&E and showing colonic inflammation in different groups: magnification is 10x; bottom panel, Alcian blue-stained goblet cells (blue): magnification is 20x. (b) Percentages of neutrophils and monocytes located in distal colons. (c) Number of goblet cells; these cells were quantified from at least 20 crypts per region in five fields in four different slides per animal. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

recruited into the colon. As shown in Figure 6(a), by day 8 after DSS treatment we detected an important systemic increase in the inflammatory monocytes CD11b^{+}Ly6^{hi}CCR2^{+} in uninfected mice, whereas mice infected with *T. crassiceps* and exposed to DSS displayed a reduced percentage of these inflammatory monocytes (Figures 6(a)-6(b)). In contrast, CD11b^{+}Ly6^{low}CCR2^{-} cells were increased in these mice (Figure 6(c)).

To further explore the effect of *T. crassiceps* infection on the development of colitis, we looked for markers of AAMΦs locally in the colon tissue. We found that colons from *T. crassiceps*-infected mice that received DSS positively expressed Arginase 1, FIZZ-1, and Ym-1 (Figure 7(a)), all molecules associated with AAMΦs. In contrast, in the same samples inducible nitric oxide synthase (iNOS) was not detected. It is interesting that uninfected mice exposed to

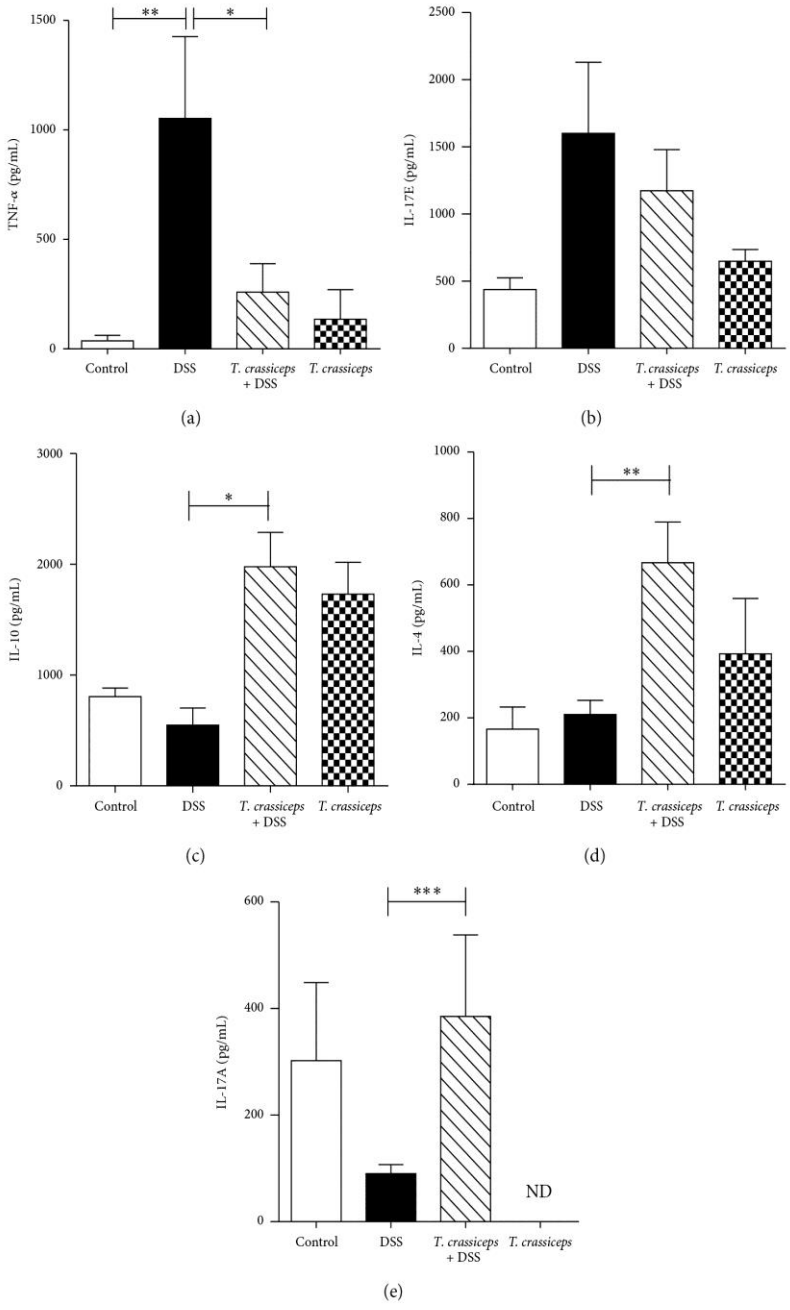

FIGURE 3: Systemic cytokine profile of *T. crassiceps*-infected and uninfected mice during DSS-induced colitis. (a) Sera TNF-α detection. (b) Sera IL-17E detection. (c) Sera IL-10 detection. (d) Sera IL-14 detection. (e) Sera IL-17A detection. Data are means ± SE and are representative of three independent experiments, $n = 4$ mice per group. $^{*}P < 0.05$ comparing *T. crassiceps*-infected mice and uninfected mice at the end of the experiment.

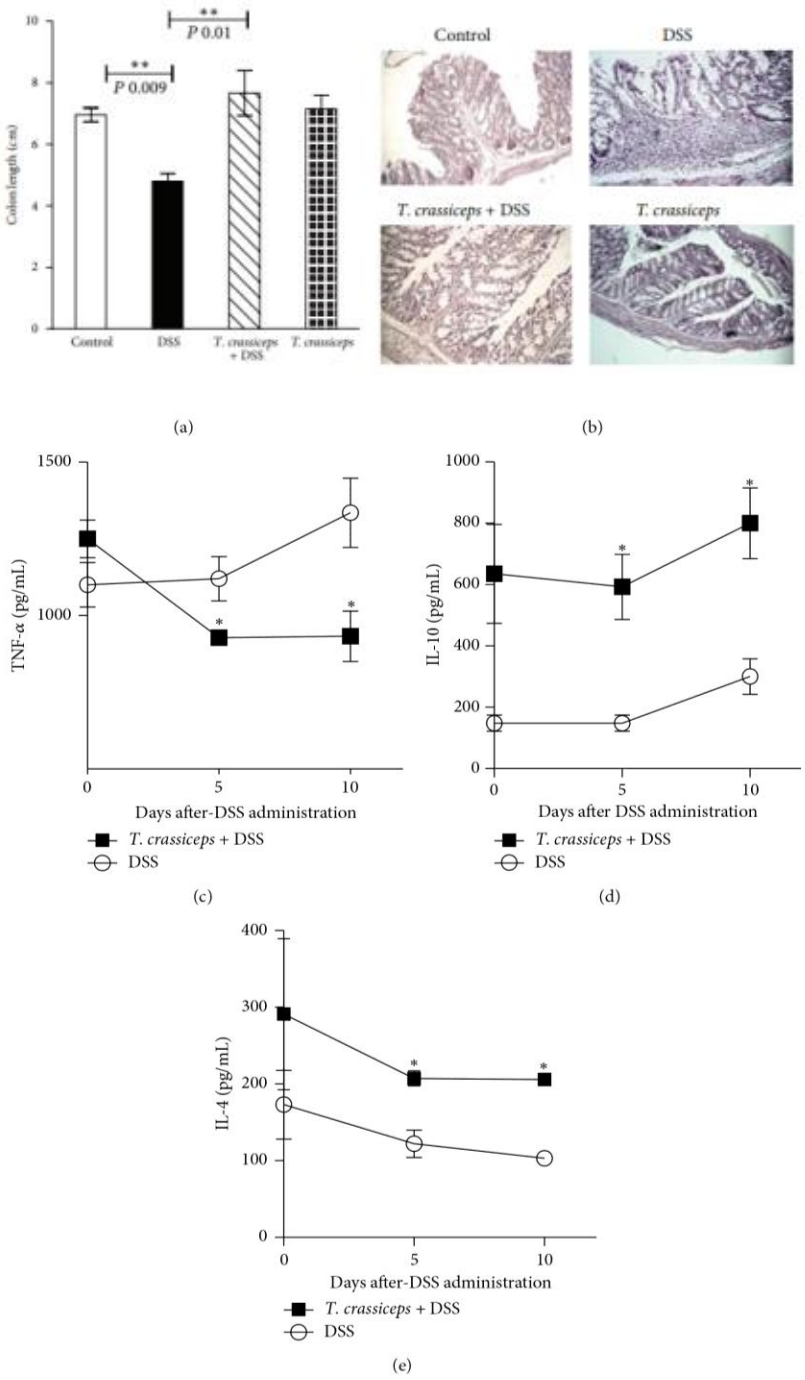

FIGURE 4: The anticolitic effect of *T. crassiceps* infection is independent of the genetic background of the host. The C57BL/6 mice were infected or not with 40 metacestodes of *T. crassiceps* and ulcerative colitis was induced. (a) Length of the colon for different groups exposed or not exposed to *T. crassiceps* infection. (b) Colon tissue histology stained with H&E and showing colonic inflammation in different groups, magnification = 20x for Control, DSS, and *T. crassiceps* + DSS, 10x for *T. crassiceps*. Serum levels of (c) TNF-α, (d) IL-10, and (e) IL-4 detected by ELISA on different days after exposure to DSS. Data are means ± SE and are representative of two independent experiments, $n = 4$ mice per group. $^{*}P < 0.05$ comparing *T. crassiceps*-infected mice and uninfected mice at the end of the experiment.

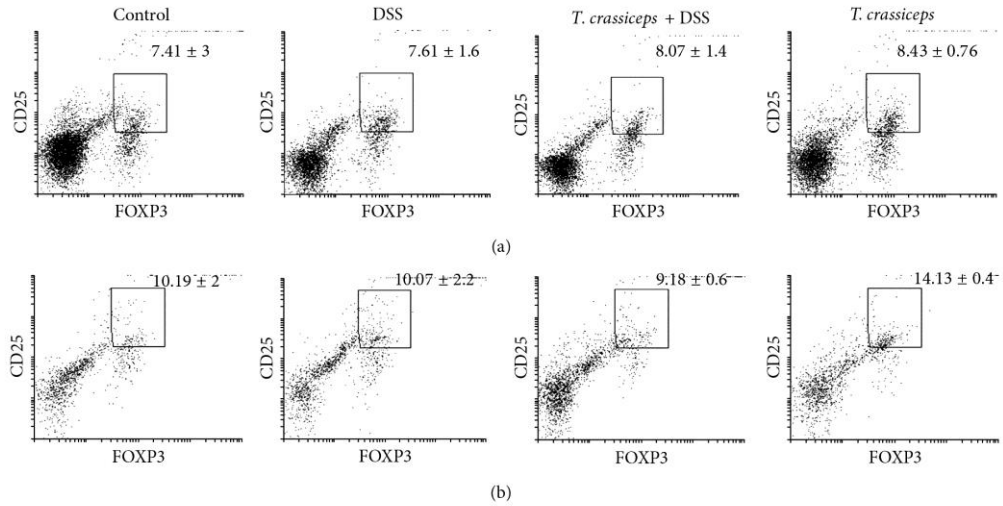

FIGURE 5: Percentages of T regulatory cells are not altered by *T. crassiceps* infection. Using the Foxp3 reporter mice C.Cg-*Foxp3*^{tm1Tch}/J, we analyzed the expression of CD25 and FOXP3 as indicative of the presence of T regulatory cells in (a) spleen cells and (b) peritoneal exudate cells. No significant differences were found among treatments. $n = 4$ mice per group.

DSS did not express Arginase-1 and Ym-1, but they did express iNOS (Figure 7(a)). Thus, the presence of *T. crassiceps* attenuated the levels of mRNA iNOS, IL-17, and TNF-α in both colon tissue and sera.

A classic side effect of strong Th2-type-biased responses induced by helminths is potential development of fibrosis [9, 27]. Here we found that *T. crassiceps*-infected and DSS-treated mice did not display an excess of collagen in the colon tissue, thus ruling out fibrosis as a side effect of this infection during the modulation of colitis (Figure 7(b).

3.5. Transfer of AAMΦs (F4/80$^+$MR$^+$) with Suppressive Activity from T. crassiceps-Infected Mice Ameliorates DSS-Induced Colitis. Based on our observation that percentages of T regulatory cells were not altered by infection with *T. crassiceps* and because *T. crassiceps* infection recruits AAMΦs into the peritoneal cavity (approximately 35% of peritoneal exudate cells are F4/80$^+$MR$^+$Arg1$^+$) and peritoneal adherent cells suppress T cell proliferation [28], we evaluated whether this population was able to influence the development of DSS-induced colitis. We obtained peritoneal cells from mice previously infected with *T. crassiceps* (6–8 weeks after infection) and sorted them in F4/80$^+$MR$^+$ (AAMΦs) and F4/80$^+$MR$^-$ with purity >90% (see Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/563425). Cells (1×10^6 F4/80$^+$MR$^+$ or F4/80$^+$MR$^-$ cells) were adjusted and immediately transferred intraperitoneally to mice previously exposed to DSS. A portion of these purified cells were tested for in vitro suppressive activity on CD4 and CD8 cells from naïve mice. As shown in Figure 8(a), the F4/80$^+$MR$^+$ cells strongly suppressed the proliferation of CD4 cells as well as CD8 cells (Supplemental Figure 2). In contrast, F4/80$^+$MR$^-$ cells were unable to inhibit T cell proliferation in response to anti-CD3/CD28 stimuli. It is important to note that the transfer of F4/80$^+$MR$^+$ cells ameliorated DSS-induced colitis by significantly decreasing bloody diarrhea (Supplemental Figure 3). Moreover, mice that received F4/80$^+$MR$^+$ cells displayed less signs of colitis, such as shortened colon, and tissue architecture was very well conserved in these mice (Figures 8(b)-8(c)) compared to mice that did not receive cells or mice that received F4/80$^+$MR$^-$ cells. The transfer of F4/80$^+$MR$^+$ cells into DSS-treated mice was characterized by much less severe mucosal pathology than in mice that did not receive cells or mice that received F4/80$^+$MR$^-$ cells, as evidenced by marked destruction of the crypt architecture and a greater influx of inflammatory polymorphonuclear cells, which are largely associated with colitis (Figures 8(c)-8(d)); thus mice receiving F4/80$^+$MR$^-$ cells showed even worse pathology, with shorter colons and severe signs of cryptitis and loss of colon tissue architecture.

3.6. In Vivo Indomethacin Treatment Impairs the Anticolitic Effect of T. crassiceps Infection. Previous work from our lab demonstrated that AAMΦs recruited by *T. crassiceps* infection are strong producers of PGE_2 [29]. However, PGE_2 may play a dual role in inflammatory processes, mainly in the gut [30]. Thus, to further elucidate the possible mechanisms involved in the effect of *T. crassiceps* on the amelioration of ulcerative colitis, we injected uninfected and *T. crassiceps*-infected mice with 3 mg/kg indomethacin daily to transiently block PGE_2 production in vivo. Injections began 2 days before the induction of colitis and were maintained throughout the experiment. Loss of body weight was significantly greater in DSS + indomethacin-treated *T. crassiceps*-infected mice than in DSS-treated *T. crassiceps*-infected mice (Figure 9(a)).

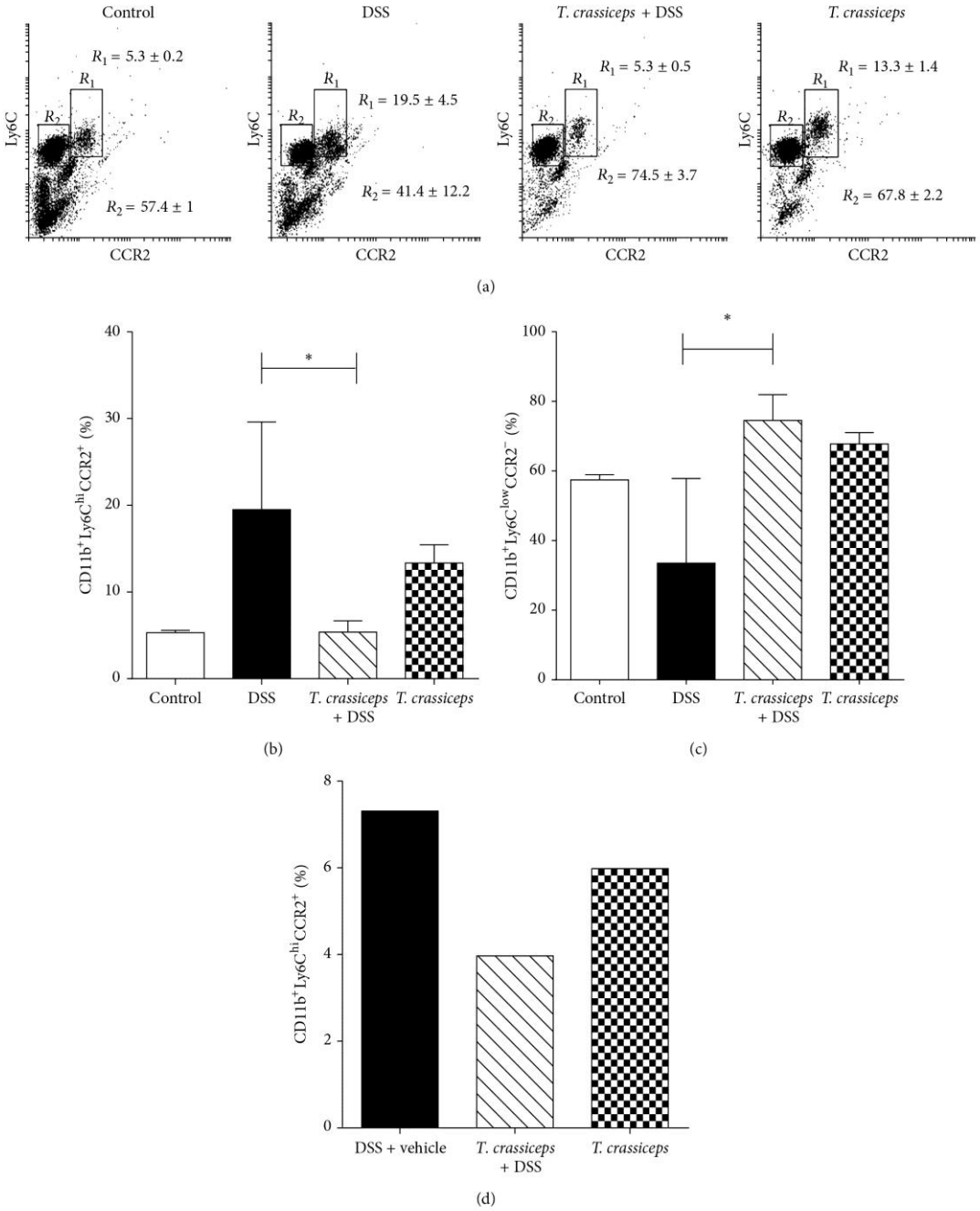

FIGURE 6: *T. crassiceps* infection reduces the number of inflammatory monocytes during colitis. (a) Representative flow cytometry plots from control mice, DSS-treated mice, and *T. crassiceps* + DSS mice gated on $CD11b^+$ living cells isolated from the circulation. Quantification of circulating (b) $CD11b^+Ly6C^{hi}CCR2^+$ cells and (c) $CD11b^+Ly6C^{lo}CCR2^-$ cells. (d) Percentage of $CD11b^+Ly6C^{hi}CCR2^+$ and $CD11b^+Ly6C^{lo}CCR2^-$ in cells isolated from the colonic lamina propria. Data are representative of two independent experiments. Values are means ± SE (n = 4 mice/group). $^*P < 0.05$, pooled cells for (d).

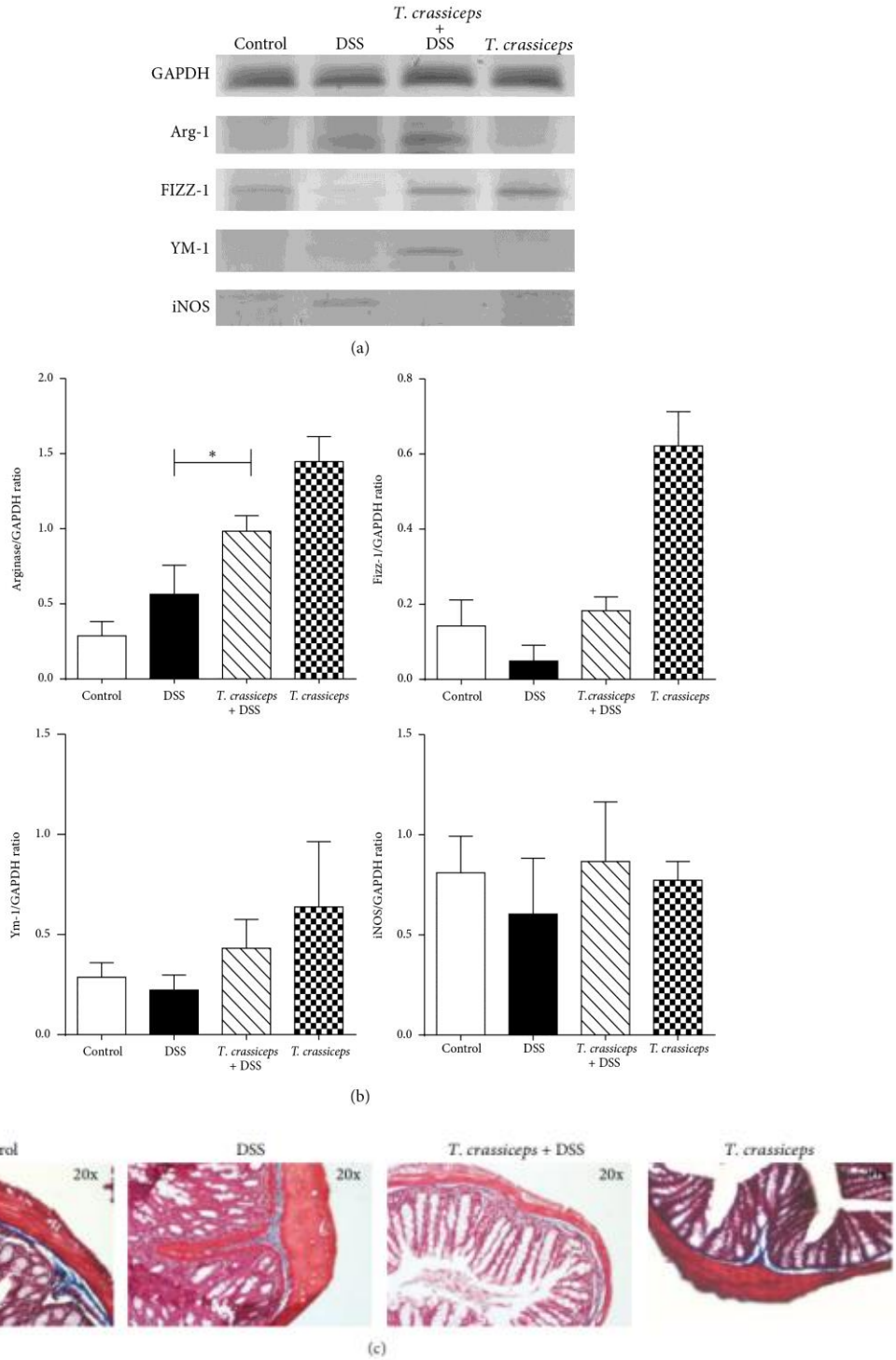

FIGURE 7: Colon tissue from *T. crassiceps*-infected mice and uninfected mice displays different levels of AAMΦ-associated transcripts. (a) Colon tissue was collected at the end of the experiments, and transcript levels of GAPDH, Arginase 1, Fizz1, Ym1, and iNOS were analyzed by RT-PCR. (b) Densitometry of Arg 1, Fizz1, Ym1, and iNOS. (c) Histology with Mason stain for the identification of collagen deposition as a sign of fibrosis. *T. crassiceps* infection does not induce fibrosis during colitis (collagen in dark blue). Magnification = 10x.

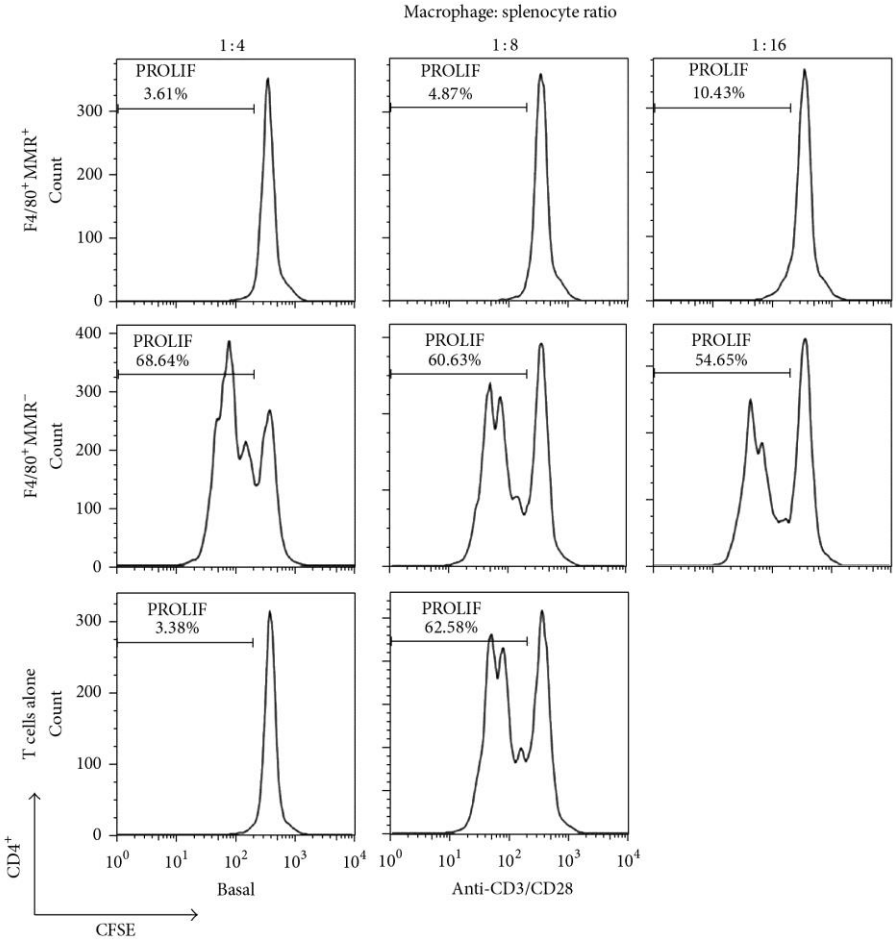

FIGURE 8: Continued.

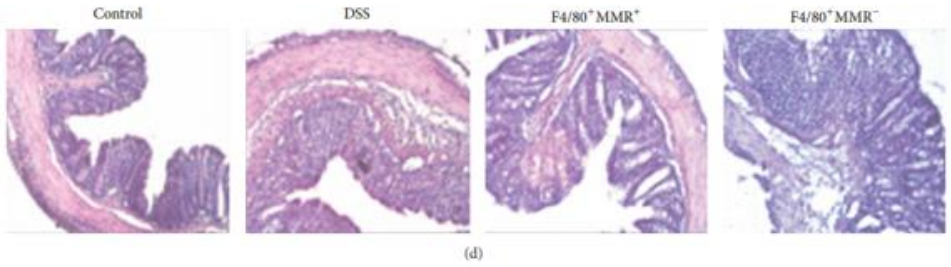

FIGURE 8: F4/80$^+$MR$^+$-sorted peritoneal macrophages from *T. crassiceps*-infected mice transferred intraperitoneally into naïve mice inhibit the development of colitis. (a) F4/80$^+$MR$^+$ peritoneal macrophages sorted from *T. crassiceps*-infected mice inhibit CD4 and CD8 T cell proliferation (data not shown for CD8). In contrast, sorted F4/80$^+$MR$^-$ macrophages from the same mice do not suppress CD4 cell proliferation. (b) Colon length of mice with ulcerative colitis that received F4/80$^+$MR$^+$ and F4/80$^+$MR$^-$ cells. (c) Infiltration of inflammation. (d) Histology of the effect of F4/80$^+$MR$^+$ adoptive transfer during colitis: magnification is 20x for all the slides shown. Bars represent the mean ± SD from three slides per mouse. $^*P < 0.05$, $^{***}P < 0.003$, $n = 5$ mice per group. All data are representative of two independent experiments.

This effect correlated with increased signs of morbidity (DAI) over the course of the disease compared to *T. crassiceps*-infected mice similarly treated with DSS (Figure 9(b)). Moreover, increased colon shortening was found in *T. crassiceps*-infected mice exposed to DSS and indomethacin compared to *T. crassiceps*-infected mice exposed to DSS alone (Figures 9(c)-9(d)). It is interesting that indomethacin treatment eliminated the pathological differences between uninfected and *T. crassiceps*-infected mice associated with the development of colitis, as shown in Figures 9(b)–9(e), as a loss of colon tissue architecture was detected even in the presence of this helminth infection. Moreover, a clear change in inflammatory recruitment was found among the different groups of mice. For example, whereas *T. crassiceps* infection reduced the influx of neutrophils into the lamina propria during colitis, uninfected mice recruited higher numbers of neutrophils. What is interesting is that blockage of PGE_2 by indomethacin treatment significantly increased the influx of neutrophils into the colon of *T. crassiceps*-infected mice (data not shown), and a significant reduction in the number of goblet cells was observed in DSS + indomethacin-treated *T. crassiceps*-infected mice, whereas mice with *T. crassiceps* infection plus colitis retained a higher number of these cells (Figures 9(e)-9(f)). Finally, mice harboring *T. crassiceps* and treated with indomethacin displayed increased production of TNF-α and IL-17 compared to *T. crassiceps*-infected mice (data not shown), whereas IL-10 was not affected either at the systemic level or in colonic extracts (data not shown).

4. Discussion

The frequency of autoimmune and inflammatory diseases such as multiple sclerosis, type 1 diabetes, and IBD has increased enormously in the past few years, a situation attributed to a lack of exposure to pathogens, especially helminths [4]. Among such inflammatory diseases, IBD has increased at an alarming rate in the past decade, accompanied by improved hygiene, sanitation, and medical conditions and, of course, less infectious diseases, including helminths [4, 11]. Symptoms of IBD are the result of complex interactions among genetic and environmental factors and the immune response [31]. Immunomodulatory effects exerted by helminth parasites on their hosts help to prevent or ameliorate such diseases [14]. Although a large body of evidence indicates that regulatory mechanisms triggered by helminth infections may help to modulate colitis, the precise mechanisms involved are not yet very well understood. Mainly gastrointestinal and transient infections of helminths induce higher levels of Th2 cytokines, induction of T regulatory cells, recruitment and expansion of AAMΦs, and reduction of inflammatory cytokines that results in amelioration in different murine models of colitis [14, 24, 32]. All of these observations suggest that distinct helminths may trigger different pathways to modulate this particular inflammatory disease.

Here we demonstrated that extraintestinal infection with the larval stage of *T. crassiceps* can be added to the growing list of helminth infections with the capacity to modulate colitis; this is just the second cestode reported to induce such protection [14]. Besides the high levels of IL-4 expected with this infection we also found elevated IL-10 levels associated with a downregulation of proinflammatory cytokines. However, *T. crassiceps* infection did not induce higher numbers of T regulatory cells, which is in line with a previous finding on the effect of this infection on experimental autoimmune encephalomyelitis [18]. Therefore, we think that T regulatory cells may play a minor role in our system, even though several authors have found that increased levels of T regulatory cells are associated with improvement in colitis during helminth infections [25, 26]. Here, using T regulatory cell reporter mice we did not find increased levels of T regulatory cells during *T. crassiceps* infection and colitis, but we did observe an improvement in colitis when mice were infected. Based on these observations, our data point to a greater role for AAMΦs (as opposed to regulatory T cells) in the anticolitic

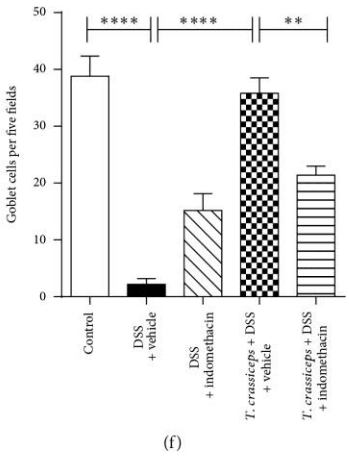

FIGURE 9: The anticolitic effect of *T. crassiceps* is abrogated by indomethacin treatment. (a) Percentage of weight loss. (b) Disease index. (c) Photograph of gross pathology of colons from different groups of mice. (d) Length of colon in infected and uninfected mice with ulcerative colitis treated or not with indomethacin (3 mg/kg). (e) Histology of colons, left panel colon tissue microphotography (10x) stained with H&E, all other panels are tissue colon stained with Alcian blue to detect goblet cells (20x and 40x, resp.). (f) Number of goblet cells for all the groups. $^{*}P < 0.05$, $^{***}P < 0.003$, $n = 5$ mice per group. Similar results were observed in two independent experiments.

effect of *T. crassiceps* infection. Furthermore, *T. crassiceps* infection in DSS-treated mice was characterized by much less severe mucosal pathology than that seen in uninfected control mice, as evidenced by marked destruction of the crypt architecture and an increased influx of inflammatory cells. Furthermore, we demonstrated that in vivo transfer of purified AAMΦs ($F4/80^{+}MR^{+}$) recruited for this infection is able to modulate ongoing colitis; thus, it is possible that these cells not only prevent the development of colitis but also may play a curative role. These observations are in line with those recently reported for another cestode, *Hymenolepis diminuta,* which induces AAMΦs associated with the prevention of colitis [9]. However, more studies are needed to confirm whether AAMΦs are the most potent immunoregulatory pathway induced by helminths to reduce colitis.

Neutrophil infiltration is a key event in inflammation of the colon [33, 34]. Here we found that *T. crassiceps* infection during DSS-induced colitis generates a significant change in the populations of cells that infiltrate the colon. We generally observed greater infiltration by monocytes than neutrophils, the latter being the main cell population detected in the absence of this helminth infection. Monocytes can be divided into two subsets: patrolling monocytes that express $CD11b^{+}CD115^{+}CX3CR1^{hi}CCR2^{lo}Ly6C^{lo}$ and inflammatory monocytes that express mainly $CD11b^{+}Ly6C^{hi}CCR2^{+}$ [35]. Inflammatory monocytes accumulate in response to infection or tissue injury, and in most cases they help to clear pathogens [36]. However, in some pathology and especially in IBD, the recruitment of inflammatory monocytes into damaged tissue frequently worsens the inflammation [35]. The recruitment of inflammatory $Ly6C^{hi}$ monocytes into adult mucosa is dependent on CCR2 expression [37]. Here we found that the circulating levels and recruitment of $CD11b^{+}Ly6C^{hi}CCR2^{+}$ inflammatory monocytes into the colon were significantly reduced by the presence of *T. crassiceps* infection; in contrast, $CD11b^{+}Ly6C^{lo}CCR2^{-}$ cells increased during infection. This is the first time that it has been reported that an extraintestinal helminth infection is able to modulate these cell populations. Such modulation may have an impact on the development of colitis and also may favor development into AAMΦs, as demonstrated by the expression of Arg 1, FIZZ 1, and YM1 in colon tissue. Thus, a reduction in the recruitment of inflammatory monocytes together with an increase in AAMΦs in the colon may be a strong anticolitic mechanism triggered by *T. crassiceps* infection.

An interesting finding is that indomethacin treatment to inhibit PGE_2 production in vivo during *T. crassiceps* infection and exposure to DSS completely abrogated the anticolitic effect of this infection. This result suggests that the in vivo capacity of *T. crassiceps* infection to suppress DSS-induced colitis is highly dependent on the ability of the host to produce PGE_2, maybe in response to commensal or parasite-derived stimulation. These findings are in line with those reported by Bao et al. [38], who found that PGE_2 plays a fundamental role in regulating the immune response to colitis as well as modulating Th1/Th2 responses [38]. Specifically, when we blocked in vivo PGE_2 using indomethacin, the anticolitic effect of *T. crassiceps* infection was clearly abrogated, but IL-10 levels still remained elevated, indicating a major role for PGE_2 during *T. crassiceps*-mediated amelioration of colitis. In line with these data, other authors have found that even in the absence of IL-10, colitis can be modulated [39]. As further support of this idea, our group previously reported that macrophages obtained from *T. crassiceps*-infected mice are able to produce significantly elevated levels of PGE_2 in response to

stimulation with LPS [29], a molecule to which epithelial and intestinal macrophages are highly exposed.

Moreover, we found the number and size of goblet cells increased in infected animals with DSS-induced colitis compare with colitic mice, suggesting that *T. crassiceps*, like other helminths, can help in preserving these cells [40, 41]. Goblet cells are involved in regulating both the mucosal barrier and the relative composition of the luminal microbiota by mucin production [42]. The high expression of IL-4 in *T. crassiceps* + DSS mice suggests that this infection may maintain the numbers of goblet cells. The production of mucus by these cells could limit bacterial access to epithelial cells and prevent chronic inflammation [43]. Thus, an increase or recruitment of AAMΦs in the lamina propria seems to be necessary for anti-inflammatory activity. We demonstrated that *T. crassiceps* infection during colitis is able to promote the polarization of AAMΦs, thereby attenuating the expression of inflammatory cytokines, preventing damage to the colon and the development of colitis. The relationship between infiltrating AAMΦs and prognosis in colitis has not been analyzed; therefore, the distribution and function of macrophages in experimental ulcerative colitis need to be evaluated further. Another interesting finding here is a trend to reduce IL-17E by *T. crassiceps* infection in colitis, but surprisingly IL-17A levels were increased in the same group; in line with these data are several findings indicating that the presence of IL-17A has an anticolitic effect, given that IL-17AKO mice became dramatically susceptible to DSS-induced colitis [44] and other researchers reported similar findings in distinct models of colitis, these data are in this moment very difficult to explain, and suggest that Th17 family has complex functions during different inflammatory diseases [45]. The mechanisms regarding the differential IL-17 modulation by *T. crassiceps* infection remain to be elucidated. Finally, the modulation of Th1- and Th17-type cytokines observed here accords with Tao et al. [22], who suggested that inactivation of STAT1 and STAT3 may contribute to resolving different models of colitis. We recently found that infection with *T. crassiceps* or its excreted/secreted products are able to inhibit STAT1 phosphorylation in macrophages and splenocytes in response to IFN-γ [46]. Thus, it appears that multiple mechanisms can be triggered by *T. crassiceps* infection or its products to modulate inflammatory responses.

5. Conclusion

We found that extraintestinal infection with *T. crassiceps* significantly reduced both symptoms and colonic inflammation associated with ulcerative colitis independently of the genetic background. Moreover, AAMΦs and prostaglandins may play a critical role in avoiding colonic inflammation and perhaps inhibiting recruitment of inflammatory monocytes $CD11b^+Ly6C^+CCR2^+$ into the lamina propria of the colon.

Conflict of Interests

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

Authors' Contribution

Yadira Ledesma-Soto and Blanca E. Callejas contributed equally to this work.

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Short Research Communication

Extraintestinal Helminth Infection Reduces the Development of Colitis-Associated Tumorigenesis

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Abstract

Colitis-associated colorectal cancer (CAC) is one of the most common cancers and is closely related to chronic or deregulated inflammation. Helminthic infections can modulate inflammatory responses in some diseases, but their immunomodulatory role during cancer development remains completely unknown. We have analyzed the role of *Taenia crassiceps*-induced anti-inflammatory response in determining the outcome of CAC. We show that extraintestinal *T. crassiceps* infection in CAC mice inhibited colonic inflammatory responses and tumor formation and prevented goblet cell loss. There was also increased expression of IL-4 and alternatively activated macrophages markers in colonic tissue and negative immunomodulation of pro-inflammatory cytokine expression. In addition, *T. crassiceps* infection prevented the upregulation of β-catenin and CXCR2 expression observed in the CAC mice, which are both markers associated with CAC-tumorigenesis, and reduced the numbers of circulating and colonic $CD11b^{+}Ly6C^{hi}CCR2^{+}$ monocytes. Thus, immunomodulatory activities induced by helminth infections may have a role in the progression of CAC.

Key words: Colitis-associated colorectal cancer, *T. crassiceps*

Introduction

Colitis associated cancer (CAC) is one of the most common malignant diseases and is the second leading cause of cancer death in several countries [1]. A relationship between inflammation and colon cancer development has been described worldwide [2]. Patients with inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis, are more susceptible to developing colorectal cancer [3].

It has been widely reported that some infectious agents, such as viruses and bacteria, can fuel or even be a cause for the development of cancer [4]. The role of parasitic infections in such field has been insufficiently studied, and scarce reports suggest that helminths can increase the risk of cancer [5]. The geographical distribution of gastrointestinal and parasitic infections demonstrated that they are widely distributed in tropical and subtropical areas with greatest numbers occurring in China, Africa, the Americas and East Asia. Concomitantly, epidemiological data provide strong evidence about the incidence of inflammatory bowel disease and colorectal cancer in the past three decades in developed countries [6], suggesting an inverse association between helminth infections and inflammatory bowel disease and colorectal can-

cer.

Nevertheless, helminth parasites have developed complex strategies to modulate the immune responses of their hosts through versatile immunoregulatory mechanisms. The capability of helminths to modulate an exacerbated inflammatory response has been used advantageously to study their effects on autoimmune diseases [7]; however, the effect of helminth infections on the development of cancer, specifically CAC, remains completely unknown. Here, we address the impact of a helminth infection on tumor development using a well-established chemical colorectal cancer mouse model.

Materials and Methods

Mice

Six to eight week old female BALB/c mice were purchased from Harlan Laboratories (México) and were maintained in a pathogen-free environment at the FES-Iztacala, UNAM animal facilities.

Parasites and infection

Metacestodes of *T. crassiceps* were harvested from the peritoneal cavity of female BALB/c mice after 2 to 3 months of infection, processed as previously reported [8] and the infection was accomplished by an i.p. injection of 20 small (2mm in diameter) non-budding cysticerci.

Development of colitis-associated colorectal cancer (CAC)

An extensively used CAC model was developed as previously described [9]. Briefly, *T. crassiceps*-infected mice (6 weeks post-infection) or age-matched uninfected mice were i.p. injected with Azoxymethane (AOM) at 12.5 mg/kg (Sigma, USA). Five days later, dextran sulfate sodium (DSS, MW: 35 000–50 000, MP Biomedicals. Solon, OH, USA) dissolved at 2% in drinking water was administered *ad libitum* for 7 consecutive days. Afterwards, the mice were maintained with regular water for 14 days and were subjected to two more DSS cycles. The mice were sacrificed on day 68; the colon was removed, weighed and submitted for macroscopic inspection, histopathological examination and mRNA expression assays. The mouse infection scheme and treatment are showed in Fig. 1A.

Histology

For histological analysis, longitudinal sections from the large intestine were immediately fixed by immersion in 10% formaldehyde dissolved in PBS, after one day the tissue was dehydrated in gradient alcohol concentrations and embedded in paraffin. Sections (5µm) were stained with hematoxilin/eosin to visualize neutrophils or with alcian blue stain to visualize the goblet cells, using an optical microscopy (Axio Vert.A1, Carl Zeiss). These cells were quantified (percentage) from at least 20 crypts per region in five fields in four different slides per animal at 400X magnification. For Immunohistochemistry staining of β-catenin and F4/80, the sections were incubated with optimal dilutions of anti-mouse beta-catenin (Gene Tex, Inc. USA) and anti-mouse F4/80 (e-Bioscience, USA) overnight at 4°C, and then developed following the conventional technique.

Colon Culture

Sections of 0.5 cm of the proximal colon were cut and washed three times with PBS. The colon fragments were placed into 24-well plate's culture in DMEM medium with penicillin and streptomycin and stimulated or not with LPS (1µg/mL) and cultured at 37°C with 5% CO_2. Supernatants were harvested 24h later and the concentration of TNF-α was determined by ELISA (Biolegend, San Diego, CA. USA).

RNA isolation and real-time PCR

RNA was isolated from colon tissues using a TRIzol extraction. Tissues were first disrupted in a tissue homogenizer (Bullet Blender®; Next Advance). Semi-quantitative RT-PCR was performed to assess the expression of mRNAs for YM-1, arginase, FIZZ1, iNOS, and CXCR2. cDNA was synthesized from the isolated RNA using a RevertAid H Minus First Strand cDNA synthesis Kit (Thermo Scientific). mRNA expression for β-catenin, IFN-γ, TNF-α, TGF-β and IL-4 was performed in a LightCycler 2.0 system (Roche Applied Science) using a KAPA SYBR® FAST One-Step qRT-PCR Roche LightCycler® (KapaBiosystems), according to the manufacturer's instructions. The oligonucleotide primer sequences are described in Table 1 and 2. All quantifications were normalized to GAPDH levels. The relative expression of the target genes was analyzed by the ΔΔCt method. Control group was used as "comparator samples" for quantification of those corresponding to test samples.

Cell Isolation and Flow Cytometry Analysis

For the isolation of the colonic lamina propria cells a standard procedure previously reported was used [10]. For flow cytometry, single cell suspensions of the lamina propria and the circulation obtained during the sacrifice were stained with anti-CD11b, anti-Ly6C (Biolegend, San Diego, CA), and anti-CCR2 (R&D Systems, USA) antibodies for 30 min at 4° C. The cells were washed twice and analyzed using the FACSCalibur system and Cell Quest software (Becton Dickinson, USA).

Table 1. Genes and their respective sequences used to determine alternative activation of macrophages by semi-quantitative RT-PCR.

Gene	Sequence	Melting Temperature
GAPDH	F-TCGGTGTGAACGGATTTGGC R-CTCTTGCTCAGTGTCCTTGC	56°
Arg-1	F-CAG AAG AAT GGA AGA GTC AG R-CAG ATA TGC AGG GAG TCA CC	54°
Ym1	F-TCACAGGTCTGGCAATTCTTCTG R-TTTGTCCTTAGGAGGGCTTCCTC	56°
Fizz1	F-GGTCCCAGTGCATATGGATGAGACCATAG R-CACCTCTTCACTCGAGGGACAGTTGGCAGC	65°
iNOS	F-GCCACCAACAATGGCAACAT R-AAGACCAGAGGCAGCACATC	60°
CXCR2	F-CACCGATGTATACCTGCTGA R-ACGCAGTACGACCCTCAAAC	63°

Table 2. Primer sequences for quantitative RT-PCR.

Gene	Sequence	Melting Temperature
β-Catenin	F-GGCCTCTGATAAAGGCAACTG R-CCGAGCAAGGATGTGGAGAG	57°
IFN-γ	F-AGCGGCTGACTGAACTCAGATTGTAG R-GTCACAGTTTTCAGCTGTATAGGG	57°
TNF-α	F-GGCAGGTCTACTTTGGAGTCATTGC R-ACATTCGAGGCTCCAGTGAATTCG	59°
TGF-β	F-GCCCTTCCTGCTCCTCAT R-TTGGCATGGTAGCCCTTG	63°
IL-4	F-CGAAGAACACCACAGAGAGTGAGCT R-GACTCATTCATGGTGCAGCCTTATCG	58°

Statistical Analysis

The data were analyzed by either a one-way ANOVA followed by Tukey´s Multiple comparisons test or by an unpaired two-tailed t test with GraphPad Prism 4 (San Diego, CA).

Results and Discussion

Helminth infection reduces CAC development

Because chronic or deregulated inflammation is a well-recognized risk factor for colorectal carcinoma development and because helminth infections are largely known to modulate inflammatory responses, this study was conducted to determine whether a helminth infection could impact the progression of CAC. During the course of the experiments, the uninfected mice (CAC group) exhibited several symptoms, such as piloerection and bloody diarrhea, whereas the *T. crassiceps*+CAC mice did not show any symptoms (data no shown). All the animals from the CAC group had diverse numbers and sizes of polypoid tumors, whereas 20% of the *T. crassiceps*+CAC mice were free from these types of lesions. CAC mice had numerous reddish polypoid tumors in the whole large intestine; macroscopic damage and pathological alterations were clearly visible. In particular, the middle area of the transverse and descending colon had numerous lesions (Fig. 1B, 1C). Indeed, after the colons were opened and washed out, multiple tumors were found in the middle to distal colon of the CAC mice (Fig. 1C). In contrast, the *T. crassiceps*+CAC mice had more normal shape colons and a significant and visible reduction in the number of tumors (Fig. 1C). The weight of the excised colons from the cecum to the rectum of the CAC mice showed a 2.5-fold of increase, whereas the *T. crassiceps* infection prevented this increase (Fig. 1D), most likely due to the lower number of tumors, as the *T. crassiceps*+CAC mice had a 64% decrease in the expected tumor formation (Fig. 1E). These data indicate that the presence of the extraintestinal larval stage of *T. crassiceps* can modulate CAC progression.

T. crassiceps infection reduces CAC-induced pathologic alterations

The histological study of the large intestine in the CAC mice without *T. crassiceps* infection revealed extensive chronic inflammation localized in the lamina propria, with numerous and large polypoid tumors constituted by well-formed glands revisted by atypical epithelial cells with large and dysplastic nucleus and numerous mitotic figures that corresponded to well differentiated adenocarcinoma (Fig 2B). Numerous neoplastic glands showed intraluminal abscess constituted by cellular debris and numerous neutrophils, a remarkable decrease of goblet cells (stained with Alcian blue, arrows) in these polypoid lesions was also observed (Fig 2B, D, E). These polypoid lesions also showed strong β-catenin immune-staining, which is a marker for abnormal cell proliferation and for tumorigenesis (Fig 2B). In contrast, *T. crassiceps*+CAC displayed lesser inflammatory infiltrate and small polypoid lesions constituted by hyperplastic epithelium with mild dysplasia and normal appearance and numbers of goblet cells, similar to the control mice (Fig 2C, D). Moreover, the colon tissue from these animals was negative for β-catenin immune-staining (Fig 2C) suggesting that these mice did not develop tumorigenesis. Immune-staining for F4/80, a macrophage marker, in colon tissue was performed. Both groups of mice were positive for this marker indicating macrophage recruitment, however, the intensity of this mark was higher in *T. crassiceps*+CAC group (Fig 2A-C), suggesting an increased recruitment of macrophages. Conversely, neutrophil recruitment was higher in the CAC group as observed by H&E histology and as is evidenced in the higher magnification photo and counting of these cells in the crypts (Fig 2B, E).

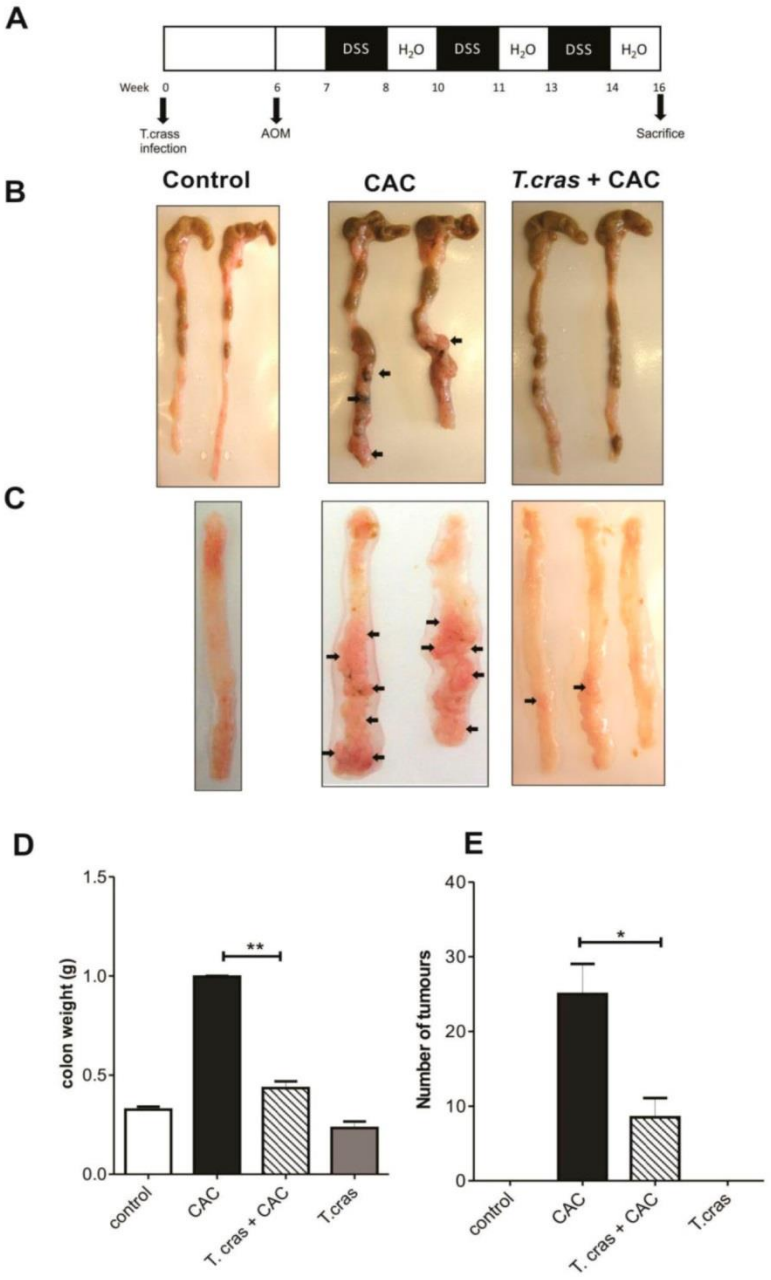

Figure 1. *T. crassiceps* infection significantly decreases colitis associated tumorigenesis. (A). Protocol for helminth infection and CAC induction. (B) Excised colon from the cecum to the rectum of the control mice, CAC mice and *T. crassiceps*+CAC mice. (C) Opened and washed colon from the proximal to the distal section of the control mice, CAC mice and *T. crassiceps*+CAC mice. (D) Colon weight after removing and flushing the cecum and rectum. (E) Tumor number count. The data are presented as the mean ± SE. Arrows show tumors. **P* < 0.05, ***P* < 0.003, n= 5 mice per group. All data are representative of two independent experiments.

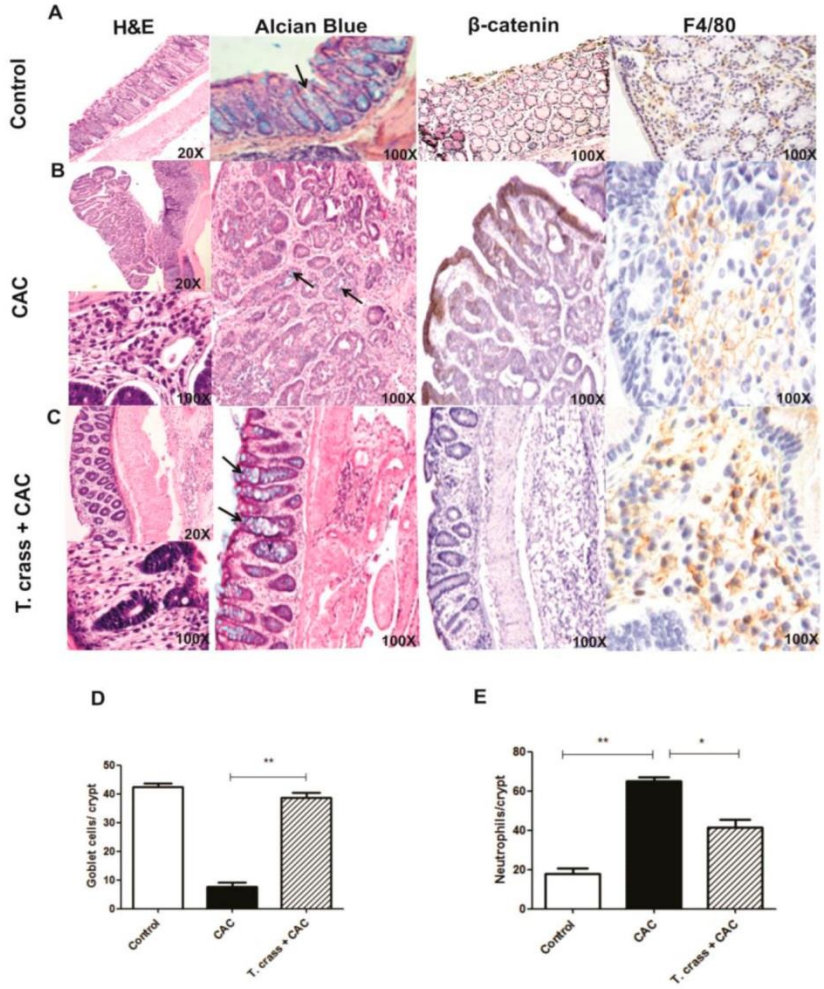

Figure 2. *T. crassiceps* infection greatly inhibits the pathology of colitis associated tumorigenesis. Representative histological features from the colon (A) control, (B) CAC and (C) *T. crassiceps*+CAC mice taken at day 68 after CAC induction and stained with Hematoxilin and Eosin (H&E), Alcian Blue for visualizing goblet cells, and immunohistochemistry stain for β-catenin and F4/80. (D) Number of goblet cells and (E) number of neutrophils located in distal colons. These cells were quantified from at least 20 crypts per region in five fields in four different slides per animal. Alcian blue stained goblet cells (arrows). Data are expressed as means ± SEM. * P< 0.05 ** P< 0.01.

Helminth infection induces differential AAMs and inflammatory marker expression during the course of CAC

We next determined the expression levels of typical molecular markers associated with both CAC development and helminth infections. We found that *T. crassiceps* infection markedly increased the mRNA expression of Ym-1, Arg-1, and Fizz-1 in the colonic tissue from the CAC-induced mice, all of these markers are characteristic of alternative activated macrophages (AAMs) (Fig. 3A-C); concomitantly, iNOS expression was significantly reduced in the *T. crassiceps*+CAC mice compared with the CAC mice (Fig. 3D). These data together with the higher number of F4/80$^+$ cells as revealed by immune-staining in the *T. crassiceps*+CAC mice suggest a preferential recruitment of AAMs induced by the presence of this helminth. We next evaluated the mRNA expression profile of IFN-γ, TNF-α, TGF-β and IL-4 in the colon. The expression of these genes was significantly enhanced, particularly TNF-α (Fig. 3F) and, to a lesser degree, IFN-γ (Fig. 3E) and TGF-β (Fig. 3G) but not IL-4 (Fig. 3H), in the CAC mice. However, in the *T. crassiceps*+CAC mice the expression of TNF-α, IFN-γ and TGF-β, was reduced, but IL-4 expression was remarkably enhanced in the colonic tissue compared

with the CAC animals (Fig. 3E-H). Thus, infection with *T. crassiceps* remarkably reduced the inflammatory infiltrate and number and size of polypoid tumors in the colon and inhibited the development of hyperplasia. This observation was correlated with attenuated production of potent inflammatory mediators, such as TNF-α and IFN-γ, both of which are known to orchestrate the development of CAC.

A feature of CAC is the chronic intestinal inflammation that mediates the breakdown of the protective intestinal barriers causing increased accessibility of the microbiota to the inflamed epithelium. This in turn may activate immune or epithelial cells through TLR-pathways, thereby amplifying the inflammatory response and generating tissue damage in the colon [11]. Excessive TLR-signaling can itself drive strong and sustained pro-inflammatory responses that fuel tumorigenesis. In this regard, *T. crassiceps* infection is able to block TLR signaling in macrophages, inhibiting IL-12, IL-23, TNF-α, and NO production, all of which are importantly involved in CAC development [12]. In order to explore whether this possibility was happening in our model we performed ex-vivo tissue colon cultures to evaluate the response to LPS. In line with this we detected an increase in TNF-α production in whole colon tissue cultures of CAC mice in response to LPS as compared with similar colon tissue cultures from *T. crassiceps*+CAC mice (Fig. 3I). These data may indicate that chronic *T. crassiceps* infection down-regulate the inflammatory response induced by LPS in vivo, suggesting that the immunomodulatory effects of this intraperitoneal infection could induce a refractory state to LPS in tissue colon during CAC development. Moreover, DCs exposed to helminth-derived products became unresponsive to TLR-stimulation in vitro [13, 14]. Indeed, *T. crassiceps* excreted/secreted products impair the pro-inflammatory response of DCs exposed to different TLR-ligands by blocking the activation of NFκB [8], an important regulator of tumor initiation and progression.

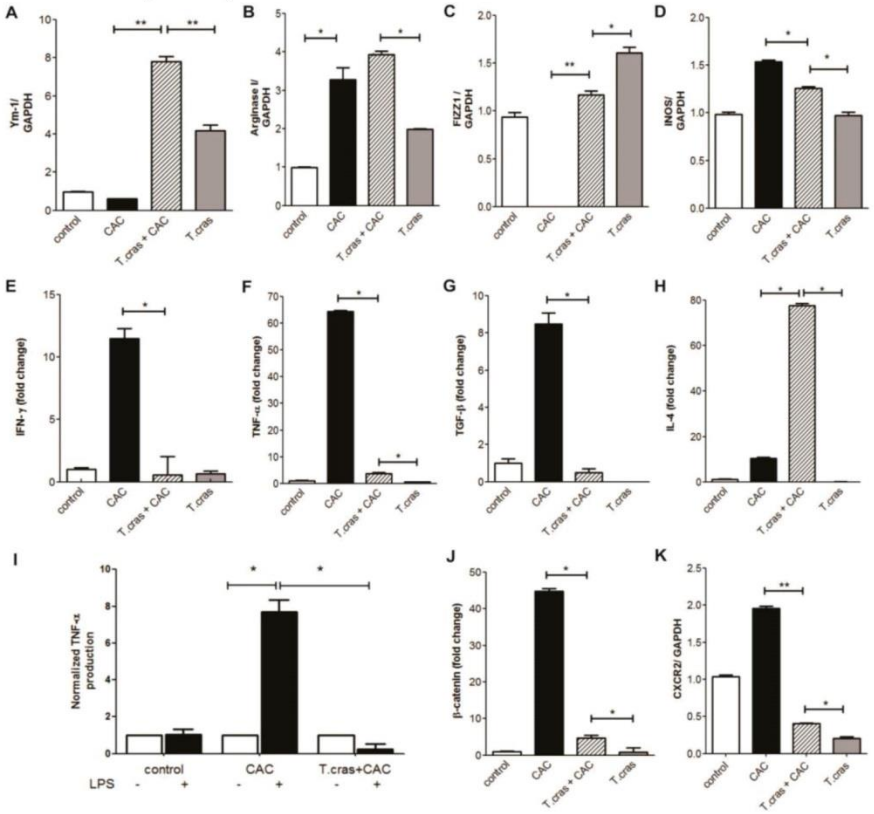

Figure 3. *T. crassiceps* infection induces a differential expression of AAM and pro-inflammatory markers during CAC. A semi-quantitative RT-PCR or quantitative RT-PCR analysis was performed on the total RNAs extracted from the colons and was normalized to GAPDH. mRNA expression of (A) Ym-1, (B) Arginase 1, (C) FIZZ1, (D) iNOS, (E) IFN-γ, (F) TNF-α, (G) TGF-β and (H) IL-4. (I) TNF-α production in ex-vivo tissue colon cultures of CAC and *T. crassiceps*+CAC mice stimulated with LPS were measured by ELISA. (J) β-catenin and (K) CXCR2 relative expression. The ratios of the mRNA levels were expressed relative to those of the mRNA levels in the untreated control group. Values are mean ± SD (n=5 mice/group) **P*<0.05, ***P*<0.03.

Conversely, IL-4 expression was enhanced locally in the colon. This cytokine may also down-regulate macrophage pro-inflammatory activation and promote AAMs. It has been well established that in addition to anti-inflammatory activity, AAMs also have the ability to repair tissue. Thus, it may be that robust early recruitment of AAMs during CAC development helps to resolve tissue damage and avoid the concomitant inflammation generated by DSS exposure thereby preventing the development of CAC. In this system, arginase 1 expression induced by the presence of *T. crassiceps* may control the harmful inflammation that occurs during CAC, consequently contributing to tissue repair in the colon and to the maintenance of the mucosal barrier. Moreover, the number and size of the goblet cells was preserved in *T. crassiceps*+CAC mice, suggesting that *T. crassiceps*, like other helminths, could increase the number of these cells [15, 16]. Goblet cells are involved in regulating both the mucosal barrier and the relative composition of the luminal microbiota by mucin production [17]. Therefore, the high expression of IL-4 in the colon of the *T. crassiceps*+CAC mice could promote the production of Th2 cytokines favoring goblet cell hyperplasia. The mucus production by goblet cells in turn may limit bacterial access to the epithelial cells and prevent chronic inflammation during CAC. Indeed, mucin 2-deficient mice develop spontaneous colitis and colonic cancers as a result of the commensal bacteria that are in direct contact with the epithelial cells [18].

We also looked for a classical molecular marker of tumorigenesis, such as β-catenin. Remarkably, we found that the CAC mice had enhanced mRNA expression of β-catenin in the colonic tissue, and importantly, the infection attenuated this increase (Fig. 3J). This is an interesting finding, as β -catenin plays an important role in cell proliferation, and its enhanced expression has been associated with colorectal carcinogenesis [19].

Given that a large number of neutrophils were observed in the colonic infiltrate in the CAC mice, we evaluated the mRNA expression of the chemokine receptor CXCR2, which is mainly expressed in these cells. CXCR2 expression was significantly increased in the CAC mice, but *T. crassiceps* infection down-regulated the expression of this chemokine neutrophil receptor in the colonic tissue (Fig. 3K). The chemokine receptor CXCR2 is a key mediator of neutrophil migration and is also involved in tumor development [20], CXCR2 is significantly up-regulated in various stages of CAC, and polymorphisms in the CXCR2 gene are associated with colon cancer progression and recurrence [21], our data here are suggestive that this helminthic infection could have potential to modulate neutrophil tumor-promoting functions.

Helminth infection prevents the increase of circulating CD11b^{+}Ly6C^{hi}CCR2^{+} and its recruitment into the colonic lamina propria

We analyzed the presence of inflammatory Ly6C^{hi} monocytes in the peripheral circulation and colonic lamina propria. Ly6C monocytes differ in their expression of a major chemokine receptor CCR2. CCR2 is responsible for the recruitment of Ly6C^{hi} monocytes to peripheral sites of inflammation. CCR2 monocyte depletion has been associated with control of inflammatory lesions, prevention of persistent inflammation and achieves control repair mechanisms [22]. CD11b^{+}Ly6C^{hi}CCR2^{+} monocytes were detected at a high frequency in the circulation of the CAC animals (Fig. 4A-B). Conversely, CD11b^{+}Ly6C^{lo}CCR2^{-} cells were significantly increased in the *T. crassiceps*+CAC mouse group (Fig. 4C). Mice in the CAC group had an increase in CD11b^{+}Ly6C^{hi}CCR2^{+} cells in the lamina propria of the colonic tissue that was blocked in the *T. crassiceps*+CAC mice (Fig. 4D). Indeed, the CAC group recruited lower percentages of CD11b^{+}Ly6C^{lo}CCR2^{-} cells into the colonic lamina propria (Fig. 4E).

Ly6C^{hi} monocytes are recruited to the inflamed colon in a CCR2 dependent fashion and give rise to pro-inflammatory effector cells that sense bacterial products and critically promote inflammation through the production of pro-inflammatory mediators [23]. The fact that *T. crassiceps* infection reduced the number of inflammatory monocytes in both the circulation and the lamina propria of the colon implies a strong regulatory activity triggered by this helminth, which could also contribute to a reduction in tumor development induced by CAC.

In sum, we demonstrate here for the first time that a helminth infection inhibits the number of colon tumors and, consequently, the development of CAC. This is most likely due to the capacity of *T. crassiceps* infection to down-modulate inflammatory responses. Several potential mechanisms may be triggered by *T. crassiceps* to inhibit tumorigenesis in this CAC model, which would be worthwhile analyzing in more detailed studies and may include: a) the recruitment of AAMs with tissue repair ability; b) lack of a response to TLR-driven inflammation; c) increase or maintenance of goblet cells and mucus production; d) blocking the recruitment of inflammatory monocytes; e) blocking of the intracellular signaling pathways associated with inflammation; and f) lowering the expression of β-catenin (proto-oncogenes). Thus, the

immunoregulatory activities of helminths may be useful in modulating the outcome of inflammatory-associated cancers in addition to their well-known role in modulating autoimmunity.

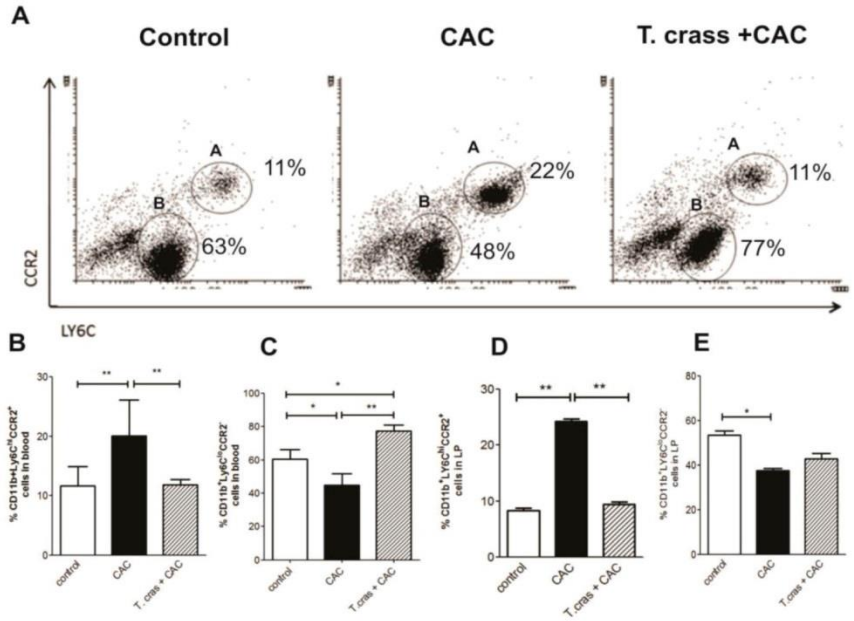

Figure 4. *T. crassiceps* infection reduces the number of inflammatory monocytes during CAC. (A) Representative flow cytometry plots from control mice, CAC mice and *T. crassiceps*+CAC mice gated on CD11b$^+$ living cells isolated from the circulation. Quantification of (B) CD11b$^+$Ly6C^{hi}CCR2$^+$ cells (region A) and (C) CD11b$^+$Ly6C^{lo}CCR2$^-$ (region B) cells from the circulation. Percentage of (D) CD11b$^+$Ly6C^{hi}CCR2$^+$ and (E) CD11b$^+$Ly6C^{lo}CCR2$^-$ in colonic lamina propria isolated cells. Data are representative of 2 independent experiments. Values are mean ± SD (n=4 mice/group) *$P < 0.05$, **$P<0.03$.

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Conflict of interest

The authors declare not conflict of interest.

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Helminth-derived molecules inhibit colitis-associated colon cancer development through NF-kB and STAT3 regulation

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Running Title

Helminth products attenuate colon tumorigenesis

Keywords

Taenia crassiceps; colorectal cancer; NF-kB, STAT3, inflammation

Novelty and Impact Statements

This study is the first to demonstrate a notable effect of *helminth-derived molecules* in suppressing ongoing colorectal cancer by downregulating proinflammatory and protumorigenic signaling such as that mediated by STAT3, AKT and NF-κB. This novel

finding supports the hypothesis that inflammation-associated signaling pathways can be re-programmed by molecules excreted/secreted by *Taenia crassiceps* (TcES) and may have important clinical implications for therapeutically targeting colorectal tumorigenesis.

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Abstract

Inflammation is currently considered a hallmark of cancer and plays a decisive role in different stages of tumorigenesis, including initiation, promotion, cancer progression, metastasis and resistance to antitumor therapies. Colorectal cancer is a disease widely associated with local chronic inflammation. Additionally, extrinsic factors such as infection may beneficially or detrimentally alter cancer progression. Several reports have noted the ability of various parasitic infections to modulate cancer development, in many cases favoring tumor progression and in others inhibiting tumorigenesis. The aim of this study was to determine the effect of excreted/secreted products of the helminth *Taenia crassiceps* (TcES) as a treatment in a murine model of colitis-associated colon cancer (CAC). Here we found that after inducing CAC, treatment with TcES was able to reduce inflammatory cytokines such as IL-1β, TNF-α, IL-33, IL-17 and significantly attenuate colon tumorigenesis. This remarkable effect was associated with the inhibition of phosphorylation of STAT3 and NF-kB. Furthermore, we determined that TcES interfered with the LPS-induced NFκB p65 activation in two colon transformed human epithelial cell lines. Moreover, in 3D cultures, TcES promoted the reorganization of the actin cytoskeleton in both cell lines, altering cell morphology and forming colonospheres, features associated with a low grade of aggressiveness. This study is the first to demonstrate a remarkable effect of helminth-derived molecules in suppressing ongoing colorectal cancer by downregulating proinflammatory and protumorigenic signaling pathways.

Introduction

Colorectal cancer is a leading cause of cancer worldwide; it is the second most common cancer in women and the third most common in men. Furthermore, colorectal cancer has the third-highest global mortality rate among cancers[1]. One variety of colorectal cancer is colitis-associated colorectal cancer (CAC), a disease with an increased chance of development in patients suffering from inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease. The development of CAC is one of the most convincing processes supporting the relationship among inflammation-dysplasia-cancer processes[2]. The inflammatory microenvironment in carcinogenesis is associated with processes involving aberrant DNA repair and excessive cell proliferation, invasion and metastasis. Reactive oxygen species (ROS), inflammatory cytokines such as IL-6, IL-1β, IL-17, and TNF-α; chemokines such as CXCL2 and CXCL5; and overactivation of various signaling pathways, including STAT3 and NF-κB, are important factors in the maintenance of the inflammatory tumor microenvironment [3].

The NF-κB protein complex is considered the master regulator of inflammatory responses; its prolonged or deregulated activation is a hallmark of inflammatory diseases and can favor tumorigenesis through the activation of proinflammatory and survival genes [4-6]. Established knowledge suggests that NF-κB crosstalk with other transcription factors and signaling proteins, such as STAT3, β-catenin and AKT, is a key promoter of colon tumorigenesis[7] .

The activation of NF-κB, STAT3, phosphoinositide 3-kinase (PI3K)/AKT and Wnt/β-catenin is not limited to cells of the immune system. In intestinal epithelial cells (IECs), constant stimulation of these pathways by activators, as well aberrant pathway activity

mediated by the loss of function of negative regulators such as SOCS3 and PTEN in the STAT3 and PI3K/AKT pathways, respectively, are key modulators of proliferation and survival [7]. Thus, in colorectal cancer, communication between the inflammatory microenvironment and IECs promotes a malignant phenotype.

Several environmental factors (chemical, physical, and biological) clearly influence cancer initiation, promotion, and progression. Regarding biological factors, infections caused by some bacteria, viruses, and protozoan and helminth parasites have been shown to be related to carcinogenesis [8]. Interestingly, the world regions with higher incidence rates of colon cancer are those associated with high standards of living, such as North America, Europe (Northern), Australia and New Zealand, where the incidence rate of parasitic diseases, especially helminth infections, is low[2].

Helminth parasites and their derived molecules can regulate several experimental inflammatory and autoimmune diseases. Several helminth parasites have been identified as promoters of some neoplasms[9]. [8, 10], while others have been reported as negative regulators of cancer development[11]. Nonetheless, the mechanisms of action triggered by parasites in modulating cancer development are diverse and incompletely described. Our previous work indicated that preinfection with *Taenia crassiceps* (a helminth) reduced the development of CAC [12]. However, the role played by molecules excreted/secreted by *T. crassiceps* (TcES) in the development of CAC is unknown. Therefore, this study aimed to evaluate the effect of TcES on CAC development and to determine the possible underlying mechanism. Treatment with TcES inhibited inflammation-associated colon tumorigenesis beginning in the early stages by regulating both STAT3 and NF-kB signaling.

Materials and Methods

Mice

Eight- to ten-week-old female BALB/c mice were purchased from Harlan Laboratories (México) and maintained in a pathogen-free environment at the Facultad de Estudios Superiores Iztacala (FES-I), Universidad Nacional Autónoma de México (UNAM) animal facilities. The animals were fed Purina Diet 5015 and water *ad libitum*. All experimental procedures were in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA) and were approved by the Committee on the Ethics of Animal Experiments of the FES-I (UNAM).

Products excreted/secreted by *T. crassiceps* (TcES)

T. crassiceps metacestodes were harvested under sterile conditions from the peritoneal cavity of female BALB/c mice after 8 weeks of infection. The cysticerci were washed 4 times in sterile PBS and cultured in PBS at 37°C for 24 h. The supernatant was recovered and centrifuged for 10 min at 5000rpm and the proteins were concentrated using an Amicon Ultrafilter with a 50-kDa cutoff membrane (Millipore, Billerica, MA, USA). The high-molecular-mass molecules were collected, and protease inhibitors were added. The samples were stored at −70°C until further use.Additionally, the endotoxin levels in TcES (<0.4 EU/mg) were evaluated using an E-Toxate Kit (Sigma, USA).

Colitis-associated colorectal cancer (CAC) model

An extensively used CAC model utilizing 12.5 mg/kg azoxymethane (AOM) (Sigma, USA), was developed as previously described (Clapper, 2007 #99). Briefly, mice received an intraperitoneal (i.p.) injection of AOM. Five days later, 2% dextran sulfate sodium (DSS, MW:40000, Alfa Aesar, Canada) in drinking water was administered *ad libitum* for 7 days. Mice were then provided regular water for 14 days and subjected to two more DSS

cycles. On day 26 after CAC induction, TcES (200 µg per mouse) were inoculated i.p. three times weekly. All mice were euthanized on day 68, the colons were removed, weighed, and submitted for macroscopic inspection and histopathological examination.

Macrophage culture and nitric oxide measurement

Single-cell suspensions of bone marrow isolated from femurs and tibias of naïve mice, were cultured in RPMI supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin and cultured at 37°C with 5% CO_2. M-CSF (10 ng ml^{-1}) (R&D Systems, UK), was added to the bone marrow cultures on days 0, 2 and 4. On day 7 cells were exposed to 25 µg of TcES for 5 minutes and then stimulated with LPS (1 µg/ml). Supernatants were harvested 24 h later, and the concentration of nitric oxide (NO) was determined using Griess reagent according to the manufacturer's instructions.

Histological analysis

For histological analysis, longitudinal sections from the large intestine were immediately fixed according to previously described protocols [12]. Colon sections with a thickness of 5 µm were stained with hematoxylin and eosin (H&E) to visualize morphology or with alcian blue to visualize goblet cells using an optical microscope (Axio Vert.A1, Carl Zeiss). For immunohistochemical and immunofluorescence staining, sections were incubated overnight at 4°C with primary antibodies against p-STAT3, p-AKT^{Ser473}, PTEN, active β-catenin, cyclin D1, c-Myc (all from Cell Signaling Technology, USA), Ki67, DNMT1, Ly6G (all from Abcam, USA), β-catenin, 8-hydroxyguanosine (both from GeneTex, USA), and BCL-2 (Biolegend, USA) and were then developed following a conventional technique. Confocal microscopy analyses were performed using a Leica TCS SP8 confocal microscope system

Colon culture and cytokine measurement

Colon culture was performed according to our previous work [12] and the concentrations of IL-1β, TNF-□, IL-10 and IL-6 were quantified by ELISA (Peprotech México, México City, México) and IL-17, IL-23, IL-31, IL-33 and ICAM-1 were quantify by Bio-Plex MAGPIX (BIO-RAD, USA) according to the manufacturers' protocols.

Gene expression

Total RNA was extracted from colon tissue using an RNA/Protein purification kit (Norgen Biotek Corp, Canada). The reactions were performed according to previous reports[14]. The specific primers for all genes are shown in Supplementary Table S1.

Cell lines and 3D cell culture

RKO cells (ATCC, Manassas, VA, USA) were cultured in EMEM medium, (Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cultures were incubated at 37°C with 5% CO_2. RKO cells were then treated with vehicle or 12.5 µg/ml TcES for 72 h and stimulated with 0.5 µg/ml LPS for 20 minutes. Then, the supernatant was collected by centrifugation at 4000 rpm for 10 minutes.

For 3D cultures, approximately 2,000 cells were plated atop reconstituted basement membrane (rBM) in 16-well slide chambers as described[15] and cultured for 10 days in the presence or absence of *T. crassiceps* antigens. The 3D structures were fixed in 4% paraformaldehyde at room temperature for 15 min. Cells were stained with Oregon Green-phalloidin and 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Confocal analyses were performed using the Leica TCS SP8 confocal microscopy system.

Proliferation and apoptosis analyses

Proliferation was measured in vitro by seeding 1×10^5 cells in six-well cell culture plates and treating them with vehicle or TcES. At specific time points, cells were trypsinized and counted using a Trypan blue (Gibco) exclusion assay.

Percent growth inhibition was calculated by normalizing the data from cells treated with TcES to that of DMSO-treated cells. IC_{50} values were calculated from dose response curves generated in GraphPad Prism using a nonlinear regression curve-fitting model.

Apoptosis was measured using an Annexin V-PE Apoptosis Detection kit (BD PharMingen) followed by flow cytometry using the FACSAria Fusion flow cytometer (BD Biosciences). A total of 10 000 cells were collected from each sample for analysis. Briefly, RKO and HCT-116 cells (2×10^5) were seeded in six-well cell culture plates and treated with vehicle or TcES for 72 h. Both floating and attached cells were collected, washed twice with cold PBS, suspended in 1× binding buffer and stained with annexin V-PE according to the manufacturer's protocol.

Western blotting assays

Total protein was extracted from colon tissues by using an RNA/protein purification kit (Norgen Biotek Corp, Canada). Additionally, protein extracts from cell lines were obtained by lysing cells with 1× RIPA buffer supplemented with complete® protease inhibitor. Proteins were separated by 10% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, USA) and blocked. The membranes were then incubated with primary antibodies against SOCS3, PIAS3, p-p65, total p65, total IκBα, p-IκBα (all from Cell Signaling Technology, USA) and β-actin (Biolegend, USA). Anti-rabbit or anti-mouse HRP-tagged secondary antibodies (BioLegend, 1:5000) were used for chemiluminescent detection with an ECL detection system (Amersham, USA).

Chemiluminescence signals were visualized on a C-Digit scanner (LI-COR Biosciences, USA), and densitometric analyses were performed with ImageJ software.

Statistical analysis

The data were analyzed with GraphPad Prism 5 by either one-way ANOVA followed by Tukey´s multiple comparisons test or by an unpaired two-tailed t test. All statistical tests were performed with 95% confidence intervals. The data are expressed as the means ± SEs, where * represents $p < 0.05$ and ** represents $p < 0.01$.

Results

Administration of helminth-derived molecules (TcES) inhibits the development of colon tumorigenesis associated with inflammation

We reported that previous extraintestinal infection with *T. crassiceps* can significantly inhibit the development of colitis-associated colorectal cancer (CAC)[12]. However, the effect of TcES during CAC development is unknown. To determine whether TcES have a protective effect on CAC progression, we used the AOM/DSS model (Fig. 1 *a*). TcES were determined to be free of endotoxin contamination (≤0.015 EU/ml) and to be biologically functional, as indicated by their ability to inhibit NO production in macrophages after strong stimulation with LPS (Fig. 1*b*). Administration of TcES was initiated on day 26 after CAC induction (CAC+TcES) and occurred three times weekly until day 68. As shown in Figure 1*c*, CAC group mice developed more than 15 tumors each in the distal and middle regions of the colon. Notably, CAC+TcES group mice developed significantly fewer tumors (3-fold less), and these tumors were smaller than those in CAC group mice (Figs. 1*d* and 1*e*). The colon weight was maintained in the CAC+TcES group (Fig. 1*f*), and approximately 45% of mice receiving TcES were tumor-free (Fig. 1*g*). However, colon tissue of CAC group mice exhibited chronic inflammation accompanied by glandular adenocarcinomas constituted by atypical epithelial cells with dysplastic nuclei and numerous mitotic figures, as well as a decrease in the number of goblet cells, whereas the colon tissue of mice treated with TcES maintained normal histology as well as unchanged numbers of goblet cells (Fig. 1*h* and 1*i*). These results suggest that TcES inhibits inflammation-associated colon tumorigenesis at early developmental stages.

Exposure to TcES inhibits STAT3 activation, reduces IL-6 receptor (IL-6R) transcription and inhibits preneoplastic cell proliferation

To gain insight into the possible mechanisms involved in the marked reduction in colon tumors induced by treatment with TcES, we evaluated the activation of protumoral signaling pathways, such as the STAT3 pathway, linked to proliferation of neoplastic cells in CAC. First, we evaluated the production of IL-6 in colon, which is a potent activator of STAT3 signaling. In colon tissue culture supernatants we found large quantities of IL-6 in both the CAC and CAC+TcES groups (Fig. 2*a*). However, STAT3 phosphorylation (pSTAT3) was inhibited in the colon tissue of the CAC+TcES group (Fig. 2*b* and 2*c*). To understand the mechanism involved in the inhibition of STAT3 activation, we next evaluated the known negative regulators of STAT3 activity, such as SOCS3 and PIAS3[16]. Expression of neither SOCS3 nor PIAS3 was significantly affected in the CAC+TcES group (Fig. 2*d* and 2*e*). To further explore how STAT3 phosphorylation was decreased in CAC+TcES group despite having large quantities of IL-6 in colon, we evaluated another important factor in the IL-6 signaling such as its receptor. Interestingly, IL-6R expression in colon extracts was significantly lower in the CAC+TcES group than in the CAC group (Fig. 2*f*), suggesting that the reduction of its receptor, could blocked up IL-6 signaling; thus, the activation of pathways associated with this cytokine, such as the STAT3 pathway, are affected. Another molecule that can be activated by IL-6 is DNMT1, a methyltransferase considered a marker in colon cancer, whose expression was also lower in the colon of CAC+TcES group mice than in the colons of CAC group mice (Fig. 2*g* and 2*h*). To verify that the STAT3 signaling pathway was affected by the administration of TcES in the CAC model, we evaluated the expression of cyclin D1, a molecule directly

dependent on STAT3 signaling pathway activation, which is essential for cell proliferation. The cyclin D1 expression was confined to the nucleus of cancer cells in the CAC group, unlike in the CAC+TcES group, in which cyclin D1 protein expression was significantly decreased in the colon (Fig. 2*i*), suggesting that IEC of CAC+TcES group mice showed lower degree of proliferation. It was corroborated through Ki67 expression (classic marker of cellular proliferation in cancer) itthat was substantially reduced in the colon tissue of animals treated with TcES (Fig. 2*j* and 2*k*). Together, these results suggest that TcES reduces IL-6R transcription, thus preventing the activation of the STAT3 signaling pathway and suppressing the proliferation of preneoplastic and transformed epithelial cells.

TcES inhibits AKT-mediated signaling and nuclear β-catenin accumulation in colorectal cancer

Several signaling pathways, such as the canonical WNT β-catenin pathway, are associated with AKT activation in colorectal cancer [17]. As mentioned above, TcES reduces the expression of IL-6R. This receptor has been reported as an activator of AKT signaling [18]. To determine whether treatment with TcES can also affect AKT activationin this model , we assessed AKT phosphorylation on serine 473 (ser473-AKT). Phosphorylated AKT was restricted to transformed colonic epithelial cells in CAC group mice; in contrast, its level was significantly decreased in CAC+TcES group mice (Fig. 3*a* and 3*b*). To determine if TcES increased the expression of negative regulators of AKT activation as PTEN, we measured the expression of this protein in colon tissue. In the figure 3*a* and 3*c* the PTEN expression was restricted to the cellular infiltrate and was reduced in the epithelial cells of CAC group mice; despite the lack of increased PTEN expression in CAC+TcES group mice compared to that in control group mice, the presence of this phosphatase was detected

in colonic epithelial cells. Due to the phosphorylation in ser-473-AKT inactivates to GSK3β and leading tothe accumulation of non-phosphorylated β-catenin in the nucleus[19], we sought to evaluate the levels of total and active β-catenin (nonphosphorylated β-catenin). The β-catenin total was mainly expressed in transformed tissue from CAC group mice, furthermore, active β-catenin was localized in the nuclei of these neoplastic cells. In clear contrast, unlike control group mice, mice receiving TcES showed basal expression of active β-catenin (Figure 3*a*, 3*d* and 3*e*). To confirm the possible downregulation of the pathways associated with β-catenin and AKT, we evaluated c-Myc expression in colon tissue. As expected, the presence of this oncogenic protein was restricted to the transformed tissue of CAC group mice, unlike in CAC+TcES group mice (Fig. 3*f*). This result suggests that TcES can prevent the activation of AKT, thus affecting the nuclear translocation of β-catenin and leading to a decrease in the expression of proteins associated with proliferation, such as c-Myc.

TcES regulates the production of oncogenic cytokines in the tumor microenvironment and the recruitment of neutrophils into the colonic epithelial barrier

To determine the effect of TcES on the inflammatory microenvironment during carcinogenesis in our model, we performed colon tissue cultures on days 26, 47 and 68 after CAC induction and evaluated the expression of cytokines with oncogenic effects in colon tissue culture supernatants and colon extracts. On day 26, increased production of IL-1β, IL-6 and IL-10 in the colon of CAC group mice, in comparison to the control group mice. After treatment with TcES on day 47, the levels of these cytokines decreased only in the CAC+TcES group. This effect was more evident on day 68, in which a significant increase in IL-1β production was observed in colon cultures from CAC mice.

(Supplementary Fig 1). Thus the most notable changes in cytokine production were observed on day 68, we evaluated the levels of other cytokines associated with tumorigenesis at this time point. To this end, we extracted protein from colonic tissue. The cytokines characteristic of Th17-type response, such as IL-17F, IL-23 and IL-33, were downregulated by exposure to TcES; in contrast, CAC group mice exhibited significantly higher levels of these cytokines (Fig. 4*a*, 4*b* and 4*c*). The level of IL-31, a cytokine associated with the regulation of cell proliferation and tissue remodeling, remained unchanged in CAC+TcES group mice relative to that in control tissue, but IL-31 expression was reduced in colons with multiple tumors (CAC group) (Fig. 4*d*). Moreover, the regulatory effect of TcES was observed in the decrease in the percentage of circulating inflammatory monocytes and the downregulation of TNF-α production but increased IL-10 levels in splenocyte cultures (Supplementary Fig. 2).

Has been reported that in the inflammatory microenvironment, in addition to cytokines, the intestinal accumulation of neutrophils contribute to initiation of inflammation-associated colon cancer, through to tissue injury[20]. Neutrophil recruitment to the site of inflammation requires the expression of CXCR2 and CXCL2 on the surface of neutrophils and in intestinal epithelial cells (IECs), respectively[21], so we evaluated the expression of CXCR2 and CXCL2 in colon tissue. Compared with CAC+TcES group, CAC group exhibited a slight tendency toward increased expression of these molecules (Fig. 4*e* and 4*f*). **Next,** we evaluated the expression of Ly6G, a marker widely associated with neutrophils, and we found that in CAC+TcES group mice, the presence of neutrophils was limited to the base of the lamina propria in the colon. In contrast, in CAC group mice, neutrophils were located close to the intestinal epithelial barrier (Fig. 4*g*). The role played by neutrophils in

inflammation-associated cancer relates to their strong capacity to produce ROS, which cause DNA damage. To evaluate the activity of neutrophils infiltrated in colon tissue, we determinate the expression of 8-hydroxyguanodsine (8-Oxo-Gua) which is a well known marker of oxidative damage (referencia Swaran JS 2014). Interestingly, despite the recruitment of neutrophils in the colon tissue of CAC+TcES group mice, the concentrations of 8-Oxo-Gua were lower than those observed in the colon of CAC group mice (Fig. 4*h*). The intercellular adhesion molecule (ICAM-1) is important for the adhesion and retention of neutrophils in the intestinal epithelium during inflammatory processes and is expressed in the apical zone [22]. In CAC+TcES group mice, ICAM-1 expression was clearly inhibited after TcES treatment compared to that in CAC group mice (Fig. 4*i*). These results suggest that administration of TcES regulates the tumor inflammatory microenvironment in CAC model.

TcES inhibits the activation of NF-κB in colitis-associated colorectal cancer..

The NF-κB signaling pathway is associated with induction of proinflammatory, proliferative and anti-apoptotic genes, and plays a critical role in the initiation and promotion of CAC, both in the tumor microenvironment and in epithelial cells. We decided evaluated if TcES administration could modified the NF-κB activation in CAC model. . In colon tissues, we assessed the phosphorylation of the p65 subunit and IκBα, the inhibitor of the p50/p65 complex.The results indicated that the phosphorylation of both of these proteins in CAC group mice was higher than that in CAC+TcES group mice (Fig. 5*a* and 5*b*). One of the receptors associated with NF-κB activation as well as other pathways related tumorigenesis is TLR4. We decided evaluated its expression in colon tissues as a possible regulation mechanism of NF-KB, triggering by TcEs treatment The results

indicated that the treatment with TcES significantly reduced TLR4 expression in colon tissue relative to that in CAC group (Figure 5*c*). This was confirmed through the TNF-α production, which is a down-stream effector in the TLR4/NF-κB signaling. Figure 5*d* shows that colon tissue from CAC group mice exhibited high TNF-α production in response to inflammatory stimulus of LPS; however, colon tissue from CAC+TcES group mice showed a marked reduction in TNF-α production in response to LPS. Another protein associated with the NF-κB activation is BCL2 (antiapoptotic protein) which also exhibited low expression in mice treated with TcES, while transformed epithelial cells from CAC group had high expression of this antiapoptotic protein (Fig. 5*e*).

To determine the direct role and possible antitumor effect of TcES in epithelial cells, we evaluated the direct effect of TcES in RKO human colon cancer cell line RKO.

Acute exposure of RKO cells to TcES did not induce a proapoptotic effect but reduced its proliferation (Fig. 5*f* and 5*g*).Furthermore, we analyzed the effect of TcES exposure in 3D cell culture conditions, notably, observed that TcES induced evident actin reorganization in RKO cell line, leading to the formation of colonospheres and preventing the classical disorganization found in transformed cell lines (Fig. 5*i*).

Discussion

Inflammation is currently considered a hallmark of cancer and plays a decisive role in different stages of tumorigenesis, including initiation, promotion, cancer progression, metastasis and resistance to antitumor therapies[9]. Additionally, extrinsic factors such as infection may beneficially or detrimentally alter cancer progression. Diverse reports have indicated a controversial role of the parasitic infections in development of cancer; either favoring or inhibiting tumor progression [8 11].. In particular, helminthic infections have been

associated with the downregulation of both inflammation-mediated diseases and inflammatory responses [9]. We have previously shown the inhibitory effect of extraintestinal preinfection with *T. crassiceps* larvae on colon tumorigenesis [12]; In contrast, other authors reported the inverse effect of a coexisting gastrointestinal helminth infection on colon cancer development [10]. These controversial reports prompted us to evaluate the role played by the *T. crassiceps*-derived products instead of a full helminth infection during the development of colitis-associated colorectal cancer (CAC). Here, we demonstrated the relevant antitumor effect of products excreted/secreted by *T. crassiceps* (TcES) on the development of colon cancer. We report a notable modulatory effect of TcES on the production of several protumor cytokines and on both NF-κB and STAT3 activation in the CAC model. Currently, the main pathological changes observed in the development of colon cancer are widely accepted to be promoted by a proinflammatory intestinal microenvironment, which, in turn, favors the activation of oncogenes[23]. In colorectal cancer, IL-6 is considered a protumoral cytokine and often correlated with poor prognosis [24]. The proliferation-and survival-promoting effects of IL-6 are mediated by the activation of the oncogenic transcription factor STAT3 and by PI3K/AKT signaling through the IL-6R and their common receptor membrane glycoprotein 130, leading to the transcription of target genes, including the cell cycle regulator cyclin D1[25]. Our results show that exposure to TcES inhibits the activation of STAT3 despite high levels of IL-6 in the colon tissue. We previously demonstrated that macrophages exposed to TcES can induce high levels of SOCS3 expression [26]; therefore, we evaluated different negative regulators of STAT3 in our system. However, the expression of neither SOCS3 nor PIAS3 by the administration of TcES was sufficient for the negative regulation of STAT3 activation. Another possible

explanation for reduced STAT3 activation could be related to the upstream IL-6R/IL-6 complex, which is required for IL-6 signaling[27]; thus, reduced expression of IL-6R results in impairedIL-6-mediated STAT3 signaling [28,29]. Accordingly, the decrease in STAT3 phosphorylation in colonic tissue after exposure to TcES appears to be mediated by a reduced expression of IL-6R mRNA. Thus, the reduction of IL-6R expression may prevent IL-6 utilization in the tumor microenvironment, leading to inhibit STAT3 activation, that could explain the reduction of tumor growth in the mice treated with TcES. Previously, has been reported in other types of cancer, such asCAC, skin cancer and oral squamous cells carcinoma, reduction of tumor growth for ablation of STAT3 and blockade of IL-6/IL-6R signaling [30, 31 32]. Therefore, our data suggest an antitumor mechanism of TcES through blockade of the IL-6/STAT3 signaling pathway, possibly inhibiting the proliferation of IECs in the CAC model.

Additionally, the decrease in AKT phosphorylation in IECs by TcES treatment in our CAC model is independent of the expression of PTEN, and could thus be a collateral event associated with the reduction in IL-6R expression. Although AKT activation can also be mediated through TLR4 and inflammatory cytokines, such as TNF-α and IL-1β[33] which were clearly reduced by TcES treatment in CAC. In addition to AKT mediated signaling, other inflammatory pathways, such as the NF-κB pathway, can lead to nuclear β-catenin accumulation in epithelial cells [5, 34], even without APC mutations [35]. As our results show, TcES inhibited the nuclear translocation of β-catenin in colon cells, as well as the expression of c-Myc in CAC model, indicating a possible mechanism by which TcES delays colon tumorigenesis via the negative regulation of AKT and β-catenin nuclear

translocation [36, 37]. Therefore, a more detailed study of the effects of the mechanisms associated with TcES signaling on AKT and β-catenin is required.

A proinflammatory microenvironment can increase the mutation rates in IECs, in addition to enhancing cell proliferation. Inflammatory cells mainly neutrophils are a major source of ROS, which can induce DNA damage and genomic instability [23]. CXCR2 is located on neutrophils and interacts with the chemokines CXCL1, -2, -3, and -5 to recruit these cells to colon tissue [38]. We found that treatment with TcES did not significantly modify the levels of CXCL2 nor CXCR2, so the percentage of neutrophils (Ly6G) infiltrating the colon did not differ from that in the CAC group. Interestingly, the localization of neutrophils was different in mice receiving TcES; neutrophils were clearly identified at the base of the lamina propria, unlike in CAC group mice, in which neutrophil infiltration was observed close to the epithelial barrier. ICAM-1 has been reported as a mediator transepithelial migration and arrest of neutrophils in the inflammatory colon [22, 39], in this context we shown a decreased of ICAM in CAC+TcES group, this could explain the lack of neutrophils migration to the apical zone in colon Another interesting finding is that ROS-induced oxidative damage was decreased due to TcES exposure, despite the presence of $Ly6G^+$ cells in the colon.

Different studies have demonstrated that blocking IL-1β or TNF-α or using IL-17 deficient mice markedly attenuates colonic inflammation and the establishment of colorectal cancer [40, 4142]. During CAC development, in addition to this constant inflammatory microenvironment and barrier dysfunction, colonic epithelial cells are exposed to a myriad of microorganisms and their products, such as LPS, resulting in prolonged activation of NF-κB signaling[23]. Consistent exposure to TcES decreased the local production of IL-1β,

TNF-α, IL-23 and IL-17, all of which are inflammatory cytokines strongly associated with fostering and exacerbating CAC development[4]. In addition to cytokines classically associated with inflammation and colon tumorigenesis, the levels of two other nonconventional cytokines, IL-31 and IL-33, appears to be modulated by TcES. IL-31 has been recently associated with intestinal tissue repair[43] and IL-33 is considered an alarmin and is released by epithelial cells after damage or necrosis[44]. thus we shown that TcES here, IL-31 production was significantly reduced in mice harboring tumors, whereas mice treated with TcES maintained normal production of this cytokine, suggesting that TcES may enhance tissue repair after AOM/DSS-induced damage. In clear contrast, IL-33 production was decreased by treatment with TcES during CAC. IL-33 is considered an alarmin and is released by epithelial cells after damage or necrosis[44]. Thus, TcES may prevent tissue damage and favor rapid tissue repair. Moreover, IL-33 induces ICAM-1 expression through NF-κB activation [45], which in turns, favors inflammation and cancer progression. Therefore, IL-33 downregulation mediated by TcES in the colon may trigger a decrease in ICAM-1 expression.

The constant activation of NF-κB in cells of the immune system augments and maintains inflammatory responses that promote cancer. However, in cancer cells, NF-κB activation stimulates proliferation, survival, invasion, epithelial-mesenchymal transition, angiogenesis and metastasis[3, 7]. Here, we found that during CAC development treatment with TcES inhibited NF-κB activation in colon tissue, possibly through decreasing TLR4 expression and suppressing the expression of downstream molecules, such as the antiapoptotic protein BCL-2. These findings may partially explain the mechanism by which TcES reduce the tumor load in CAC.

To further investigate the ability of TcES to reduce colon tumorigenesis at early stages, we extended the analysis to completely transformed human colon cancer cells.

We determined that products derived from a helminth inhibited NF-κB activation in transformed human epithelial cells. Noticeably, RKO cells exposed to TcES exhibited strikingly reduced NF-κB phosphorylation in response to LPS stimulation, confirming the blockade of NF-κB signaling. NF-κB influences the activity of the Wnt/β-catenin signaling pathway, which could induce alterations to the actin cytoskeleton, conferring migration ability on cancer cells[37, 48, 49]. We found that in 3D cultures, TcES promoted the reorganization of the actin cytoskeleton RKO cells, altering cell morphology and forming colonospheres. As previously reported in other cancer models, tumor cell reorganization is associated with a low grade of aggressiveness[49]; thus, our data suggest a regulatory effect of TcES on signals associated with migration and invasiveness.

Overall, our current study demonstrates that helminth-derived molecules downregulate the transduction of proinflammatory and protumor signals through suppressing STAT3 and NF-κB signaling, preventing colon tumorigenesis. An integrated schematic illustrating the mechanism by which TcES may block several pathways in CAC development is provided as Figure 6. Importantly, the effects of TcES translate to the human setting, as TcES inhibits both the maturation of inflammatory human DCs in response to LPS[13, 49] and STAT1 activation in human macrophages in response to IFN-γ[26]. Our data show that TcES prevented NF-κB activation in response to LPS in a transformed human colonic epithelial cell line. Therefore, TcES are likely a promising antitumor adjuvant therapy for inflammation-associated carcinogenesis.

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Conflict of interest

"The authors declare that they have no competing interests"

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FIGURE LEGENDS

Fig. 1 TcES inhibit colon tumorigenesis. (*a*) CAC induction model and administration of TcES during the development of CAC. (*b*) Production of nitric oxide in supernatants (24 h) of bone marrow-differentiated macrophages stimulated or not stimulated with LPS and TcES, was measured by the Griess reaction. (*c*) Image of colons opened longitudinally showing macroscopic aspects after the induction of CAC and administration of TcES on day 68. (*d*) Tumor loads. (*e*) Histogram showing the tumor size distribution. (*f*) Normalized weights of 5-cm colon sections. (*g*) Percentage of tumor-free mice. (*h*) Colon sections stained with H&E as well as with alcian blue to visualize goblet cells in the colon. (*i*) Graphical representation of the number of goblet cells. In all the experiments the samples evaluated were the Control, CAC and CAC+TcES. The data are expressed as the mean ± SE from 4 mice per group and are representative of four independent experiments. *** $P<0.0001$, ** $P<0.01$, * $P < 0.05$.

Fig. 2 Administration of TcES suppresses STAT3 activation and the colonic epithelial cell proliferation. (*a*) IL-6 production in colon cultures was determined by ELISA. (*b*) Representative immunohistochemical staining of phosphorylated STAT3. (*c*) Percentage of positive cells of STAT3 phosphorylation. (*d*) Representative western blot of SOCS3 and PIAS3 expression. (*e*) Densitometric analysis showing data from four western blots from independent experiments. (*f*) Relative expression of IL-6 in colon tissue by qPCR. (*g*) and (*h*) Immunohistochemical staining and Percentage of positive cells of DNMT1. (*i*) Immunofluorescence staining of colon tissue using an anti-cyclin D1 antibody (red) and DAPI (blue) counterstaining; scale bars = 20 μm. (*j*) and (*k*) Immunohistochemical

staining and percentage of positive cells of Ki67. The data are expressed as the mean ± SE from 4 mice per group and are representative of four independent experiments. ** P <0.01, *P < 0.05.

Fig. 3 TcES inhibits AKT activation and nuclear translocation of β-catenin in colonic epithelial cells in CAC. (*a*)Immunohistochemical staining of p-AKT^{Ser473}, PTEN, β-catenin and active β-catenin in colon tissue. The arrowheads indicate the nuclear localization of active β-catenin in Control, CAC and CAC+TcES samples. (*b-e*) Percentage of cells staining positive for AKT^{Ser473}, PTEN, β-catenin, and active β-catenin. (*f*) Confocal representative merge image of immunofluorescence staining of colon tissue using anti-c-Myc antibody (green) and DAPI (blue) counterstaining; scale bars = 20 μm. The results are representative from 4 mice per group of four independent experiments and are expressed as the mean ± SE. ** P <0.01, *P < 0.05.

Fig. 4 TcES regulates the production of oncogenic cytokines in the tumor microenvironment and the recruitment of neutrophils into the colonic epithelial barrier. (*a-d*)Magpix analysis was carried out on day 68 after AOM/DSS induction to measure IL-17F, IL-23, IL-33 and IL-31 levels. (*e-f*) Relative expression of CXCL2 and CXCR2 were analyzed by qPCR. (*g*) The distribution of neutrophil (Ly6G) infiltration was observed by immunohistochemical staining. The arrowheads indicate Ly6G localization. Confocal representative merge image of immunofluorescence of 8-Oxo-Gua (red) counterstained with DAPI (blue). (*h*) Levels of ICAM-1 in the all groups. The data are expressed as the mean ± SE from 4 mice per group and are representative of four independent experiments. *** P<0.0001, ** P <0.01, *P < 0.05.

Fig. 5 TcES inhibits the activation of NF-κB in colitis-associated colorectal cancer and a human colon cancer cell line. (*a* and *b*) Representative western blot and densitometric analysis showing the protein levels of phosphorylated and total p65 and IκBα. (*c*) Relative expression of TLR4 mRNA in colon tissue. (*d*) TNF-α production in ex vivo colon tissue cultures stimulated with LPS was measured by ELISA. (*e*) Confocal microscopy analysis of representative merge immunofluorescence staining of BCL-2 (green) and cell nuclear staining with DAPI (blue); scale bars = 20 μm. (*f*) Cell viability percentage as evaluated by annexin V staining in the RKO (white bars) (black bars) cell line acutely exposed to vehicle or TcES. (*g*) Cell proliferation in the human colon cancer cell lines RKO after TcES treatment (12.5 μg/ml) for 72 h exposed to vehicle (black lines) or TcES (red lines). (*h*) Representative western blot of total and phosphorylated NF-κB in the cell line RKO after acute exposure to TcES with or without 20 minutes of LPS stimulation. (*i*) Confocal representative image of immunofluorescence staining of 3D cultures of human colon cancer cell lines with and without TcES exposure; actin was labeled with Alexa Fluor 488, and DAPI was used to visualize nuclei. The data are representative of four independent experiments and are expressed as the mean ± SE. *** P<0.0001, ** P <0.01, *P < 0.05.

Fig. 6 Hypothetical model of signaling pathway regulation triggered by TcES exposure in CAC. TcES can be recognized by several pattern recognition receptors (PRRs), including TLR2, MGL and MR; after TcES binding to these putative receptors, the resulting signaling decreases IL-6 receptor expression and inhibits STAT3 phosphorylation, leading to a reduction in cyclin D1 expression and, therefore, to the inhibition of cell proliferation. Also, TcES inhibits the decrease in PTEN expression, possibly implying a

reduction in *Ser473-AKT phosphorylation* and leading to a decrease in DNMT1 expression and a possible reduction in its methylation activity. Regarding the association of β-catenin and AKT, TcES prevents the nuclear translocation of β-catenin, thus inhibiting the expression of c-Myc, a gene induced by β-catenin activity with the TCF/LEF complex. In addition, TcES may regulate TLR4 expression, leading to inhibited phosphorylation of the IκBα regulator, in turn preventing the release and nuclear translocation of the p50/p65 complex and resulting in decreased expression of the antiapoptotic protein BCL-2. Finally, in human colon cancer cell lines, we confirmed the inhibition of NF-κB activity by TcES exposure.

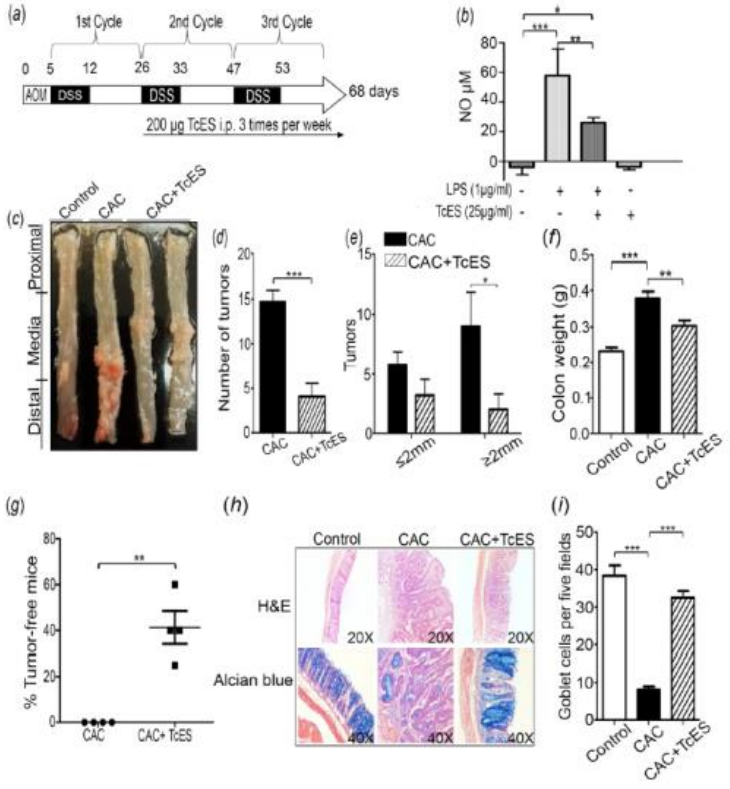

Figure 1

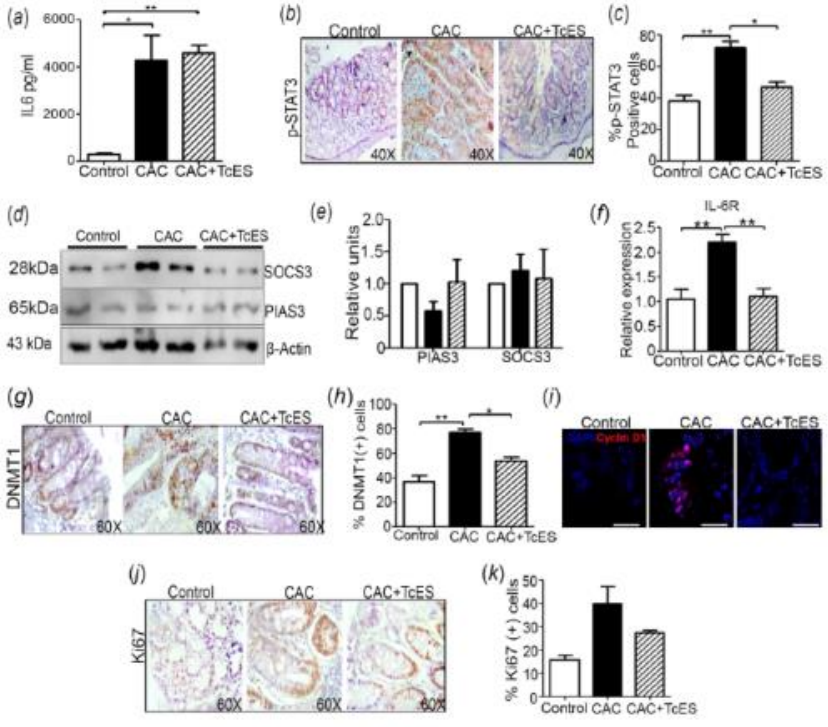

Figure 2

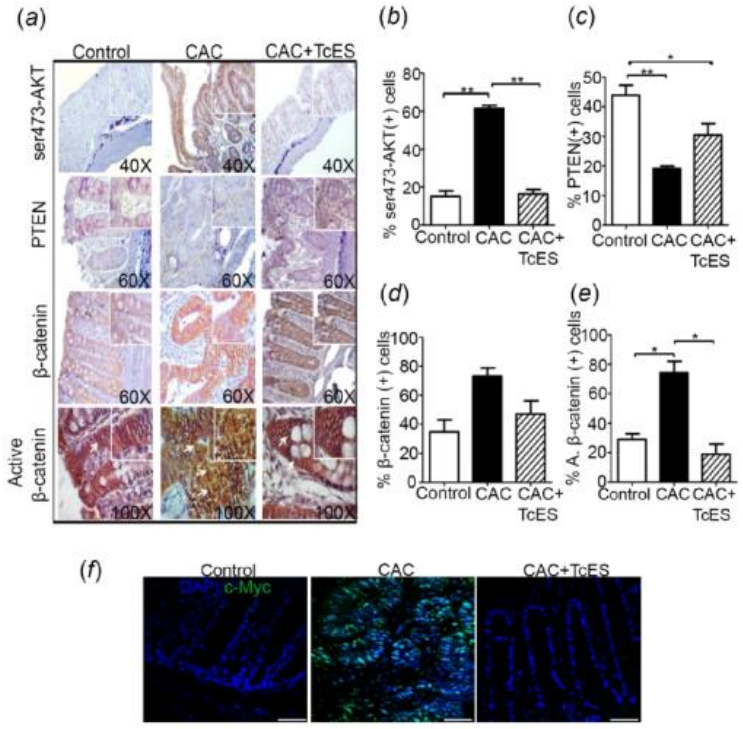

Figure 3

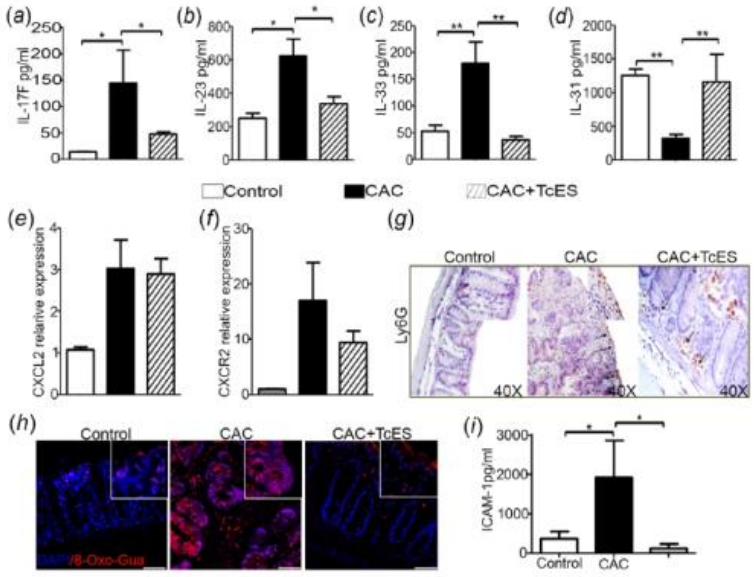

Figure 4

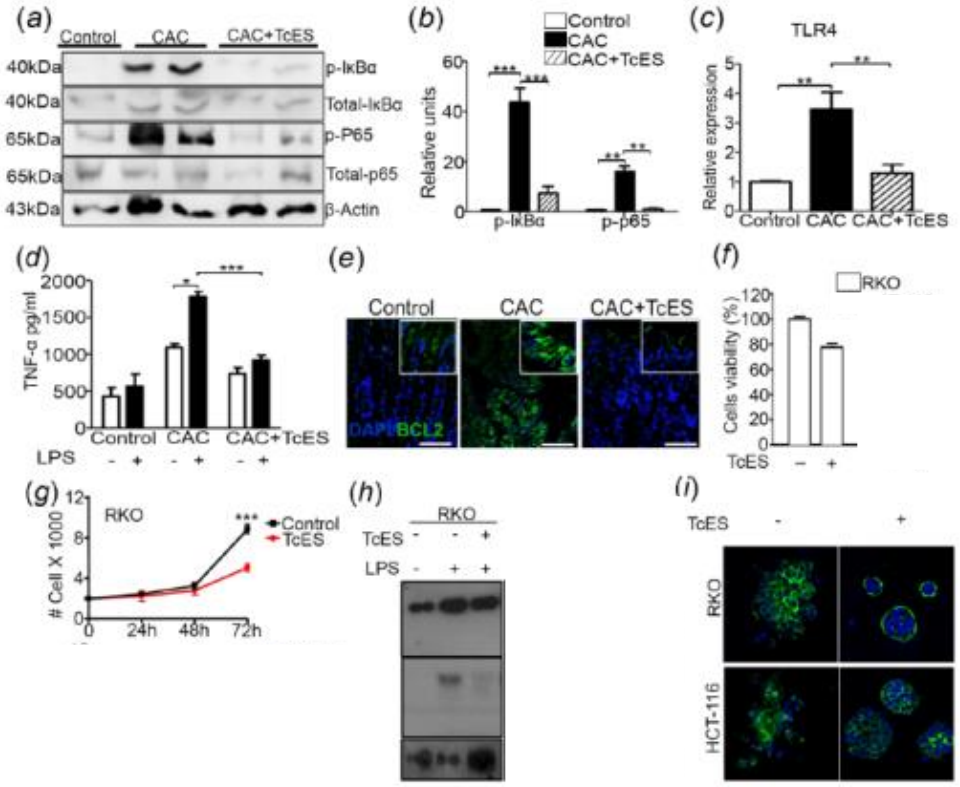

Figure 5

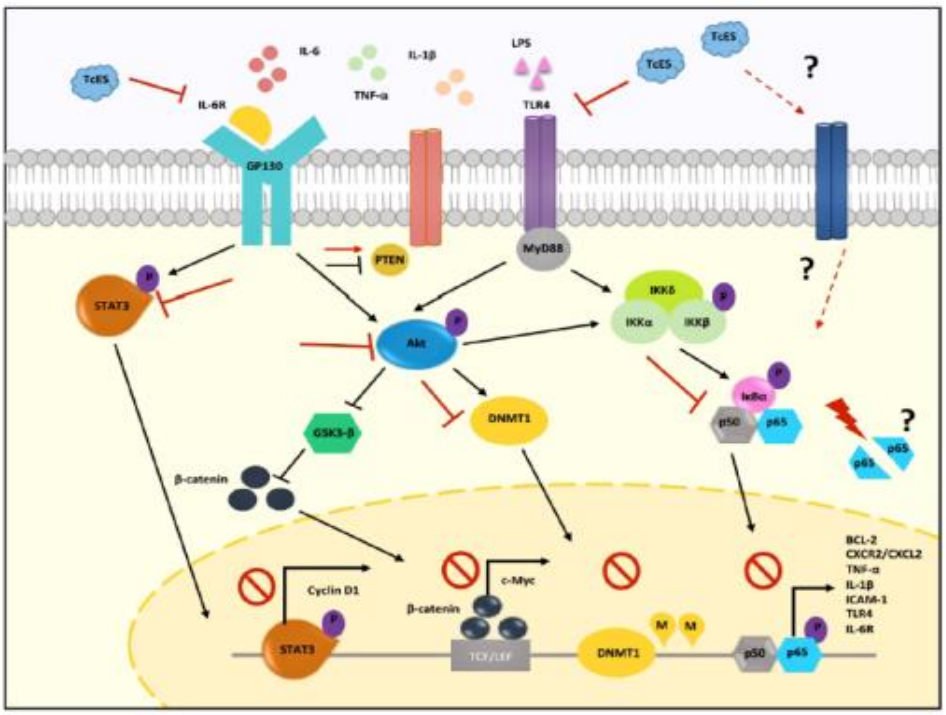

Figure 6

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Review Article

Parasites as negative regulators of cancer

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Several environmental factors (chemical, physical, and biological) can cause the initiation, promotion, and progression of cancer. Regarding the biological factors, several studies have found that infections caused by some bacteria, viruses and protozoan, and helminth parasites are related to carcinogenesis. However, in recent years a different approach has been implemented on the antitumor impact of parasitic diseases caused by some protozoan and helminths, mainly because such infections may affect several hallmarks of cancer, but the involved mechanisms still remain unknown. The beneficial effects reported for some parasitic diseases on tumorigenesis range from the induction of apoptosis, activation of the immune response, avoiding metastasis and angiogenesis, inhibition of proliferative signals, to the regulation of inflammatory responses that promote cancer. In this work, we reviewed the available information regarding how parasitic infections may modulate cancer progression. Despite the fact that specific mechanisms of action on tumors are not yet totally clear, we consider that detailed studies of the antitumor action of these organisms and their products could lead to the discovery and use of new molecules from these biological agents that may work as adjuvant therapy in the treatment of various types of cancer.

Introduction

Cancer is a set of diseases that are acquired during the development of the neoplastic cell [1,2] that shares characteristics such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, genome instability, inflammation, reprogramming of energy metabolism, and evading immune destruction. Carcinogenesis is multifactorial, where genes, microenvironment and lifestyle, among others, play a key role in the development of cancer. In addition, infectious diseases participate in modulating carcinogenesis. It has been largely known that viral infections are associated with several types of cancer (i.e. papilloma virus and cervical cancer), as well as bacterial infections (i.e. *Helicobacter pylori* and gastric cancer). Furthermore, parasitic diseases may play an important role in favoring carcinogenesis, for example, the *Schistosoma haematobium* infection is associated with cancer of the urinary bladder and the *Clonorchis sinensis* and *Opisthorchis viverrini* food-borne liver flukes are associated with cholangiocarcinoma of the liver; the latter have been classified as carcinogenic agents [3]. The role played by some parasitic diseases caused by protozoan or helminth parasites as inducers or promoters of cancer has been meticulously described recently [4]; however, its regulatory effect on tumorigenesis has received much less attention. In the present review, we have compiled a series of studies pointing out for a potential positive modulatory effect of several parasitic diseases on tumorigenesis by having an effect on several hallmarks of cancer.

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Different hallmarks of cancer are impacted by parasites and their products

Hallmarks of cancer were defined some time ago and are well-known to include critical 'factors' that contribute to the immortality of neoplastic cells (Figure 1). Some of these hallmarks are affected by parasitic

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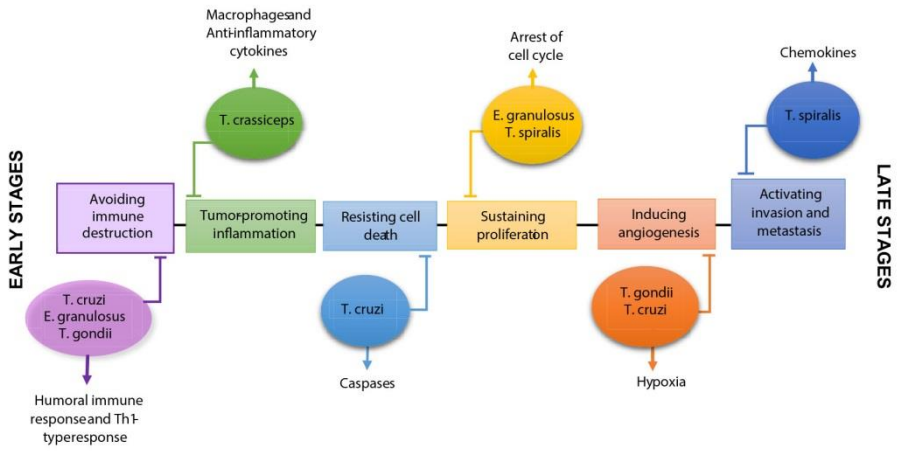

Figure 1. Parasites with therapeutic targeting of hallmark of cancer

Protozoa such as *Toxoplasma gondii* and *Trypanosoma cruzi* have an antitumor effect on some cell types of cancer through the antiangiogenic capacity, reactivation of the immune response and induction of apoptosis. On the other hand, *Taenia crassiceps* is able to regulate the cancer-promoting inflammatory response. *Echinococcus granulosus* have different antitumor mechanisms such as reactivation of the immune response and antiproliferative effect on transformed cells, as well as *Trichinella spiralis* with regulating effect of invasion and metastasis and antiproliferative signals.

infections by modifying the immune response and, in consequence, altering the immune microenvironment of the tumor. For example, a dominant Th1-type response is displayed by the host during protozoan infections, whereas a dominant Th2-type response prevails in the host during helminth infections. Both of these responses will have an effect at least in two hallmarks of cancer, such as immune surveillance and inflammation. Thus, in this study we aimed at compiling several investigations that outline an important contribution of parasitic diseases and their products on modifying either positively or negatively some of the hallmarks of cancer. In this review, we focused on reports that suggest an inverse relationship between infections by some parasites and cancer, as well as its powerful therapeutic effect on modulating different hallmarks of cancer.

Targeting hallmarks: avoiding immune destruction

The immune surveillance theory proposes that cells and tissues are constantly monitored by an immune system that is always alert; immune surveillance is responsible for detecting and eliminating preneoplastic and neoplastic cells. However, some cancer cells are able to evade the immune attack and its elimination [2]. The existence of immune surveillance has been demonstrated by the increase in the incidence of some types of cancer in immunocompromised patients, or in various animal models with elimination of one or more elements of the immune system. Due to the great importance of the immune system in the development of cancer, there has been attempts to develop immunotherapies directed against tumors with the aim of increasing the antitumor immune response and consequently the eradication of the neoplastic in progress. Among antitumor therapies, the use of helminths and protozoa for the reactivation of immune responses has been reported; such is the case of *Echinococcus granulosus*, *Toxoplasma gondii*, and *Trypanosoma cruzi*. It has been stated that different stages of the helminth parasite *E. granulosus* show antigenic similarity to mucin peptides and cancer cells, which is why several studies suggest the use of *E. granulosus* extracts as a potential inducer of antitumor activity for example, on increased activity of Natural Killer (NK) cells [5]. In addition, in an orthotopic model of colon cancer with the direct inoculation of the CT26 neoplastic cell line, prophylactic treatment based on the injection of hydatid fluid of *E. granulosus* generates antibodies capable of recognizing mortalin and creatine kinase M-type expressed on cancer cells, preventing in this way the establishment of carcinogenic cells and, therefore, tumor growth [6]. Other models described with the use of the extracts of this parasite will be mentioned later, since other antitumor mechanisms have been reported in addition to the activation of the immune response after exposure to some cancer cell lines.

Another parasite associated with an antitumor effect is *Trypanosoma cruzi*, which is a protozoan that causes Chagas disease. However, not all the information provided by this protozoan is negative, since some epidemiological studies report a lower incidence of colon cancer in patients infected with *T. cruzi* [7]. Several reports suggest that either the injection of *T. cruzi*-derived molecules or the infection with this parasite generated resistance to the development of some types of cancer. For example, in the orthotopic models of experimental breast and colon cancer, vaccination with epimastigotes of *T. cruzi* inhibited carcinogenesis through the activation of $CD4^+$ and $CD8^+$ cells, as well as by the increase of macrophages and dendritic cells, thus displaying greater NADPH oxidase activity. Also, antibodies directed against *T. cruzi* were able to specifically recognize human breast and colon cancer cell lines. Interestingly these antibodies also recognized 68% of tumor biopsies from breast and colon cancer patients [8]. Other reports suggest the use of the recombinant calreticulin of *T. cruzi* during the development of experimental breast adenocarcinoma, which reveals the presence of tumor cells to the immune system [9]. There are reports of other protozoa and their dual effect on tumorigenesis, which included epidemiological studies and *in vivo* models. *Toxoplasma gondii* is a protozoan parasite that induces strong polarization of Th1 responses in its host, with an increase in IFN-γ and IL-12 production, which is essential for resistance to this intracellular pathogen [10,11]. Due to the ability of *T. gondii* to modify the immune response of its host with this immunological profile, this parasite has been suggested as a potent inducer of antitumor responses. Therefore, the intratumoral administration of an attenuated strain of *T. gondii* in a melanoma model induced an immunogenic effect capable of stimulating the antitumor immune response, mediated by $CD8^+$ T cells and NK cells, as well as increased expression of MHC-I and MHC-II molecules on antigen-presenting cells (APC) [12]. In addition, mice immunization with dendritic cells matured in the presence of *T. gondii*-derived profilin-like protein increased the activity of cytotoxic T cells and consequently, a decrease in the melanoma and fibrosarcoma tumor growth [13].

Therefore, some molecules constituting the surface of these parasites can induce the production of antibodies that recognize tumor cells due to the antigenic similarity between them, or they can serve as activators of cells involved in the process of cancer cells recognition. In addition, some infections by themselves can promote the antitumor response, such is the case of *T. gondii*. As mentioned above, it is characterized by the induction of IL-12 production, which in turn can stimulate NK cells and T cells to produce IFN-γ [14], regulating the expansion of CD8 T lymphocytes, as well as their cytotoxic capacity; therefore, promoting the activation of an antitumor immune response.

However, an epidemiological study conducted in a Chinese population suggests a general seroprevalence of *T. gondii* in patients with cancer, compared with those who did not suffer from this condition [15]. *T. gondii* DNA was detected in the transformed cells in two patients with primary intraocular B-cell lymphoma, but not in healthy tissue cells [16]. *T. gondii* tachyzoites were also detected in bronchoalveolar lavages in a patient with squamous carcinoma [17]. Another patient with anaplastic large cell lymphoma was diagnosed with active Toxoplasmosis [18], and similar data were observed in brain cancer. However, it is hypothesized concerning the latter that *T. gondii* potentially increases the risk of this neoplasia in humans through the inflammatory and antiapoptotic response generated by its encystment in the host brain [19]. Various reports regarding the effect of this protozoon on the development of certain types of cancer are still controversial. There is not enough information regarding a cause–effect relationship between *T. gondii* and carcinogenesis, so the correlation is poor and further information is needed to clarify them.

Targeting hallmark: tumor promoting inflammation

At the beginning of the 19th century, Rudolf Virchow identified the accumulation of leukocyte infiltrate in samples of neoplastic tissue. This discovery marked the beginning of the possible association between inflammation and cancer [20]. Currently, several reports confirm a close relationship between inflammatory processes, proliferation, survival, and migration of cancer cells, as well as promotion of the release of agents that induce DNA damage [21,1]. In colitis-associated colorectal cancer (CAC), chronic inflammation plays a major role as an inducer and promoter of the neoplasm. At present, it is clearly recognized that patients with inflammatory bowel diseases as ulcerative colitis have an increased risk to develop CAC [22]. In accordance with Globocan 2012, colorectal cancer is the third most common cancer in men and the second in women worldwide [23]. To improve the study and provide new information regarding the advance and understanding of this increasing pathology, Tanaka et al. [24] developed a powerful and reproducible initiation-promotion model of colorectal cancer by using a chemical inducer of DNA damage, such as azoxymethane (AOM) followed by the exposure to an inflammatory chronic stimulus, such as Dextran Sodium Sulfate (DSS) that resembles colitis. Thus, the AOM/DSS model thoroughly resumes what happen during the process of colon carcinogenesis in humans [25]. This is a better model compared with orthotopic models that directly inject colon cancer cells subcutaneously into a tissue anatomically different from that where this cancer normally develops, given that all the process of DNA damage, inflammation, among others are lost. Taking advantage of the

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Table 1 Parasites with putative antitumor activity

Parasite	Cancer	Mechanism of action	Reference
Echinococcus granulosus	Breast and colon cancer	Production of antibodies for the recognition of tumor cells	[5,6]
	Fibrosarcoma	Not clear	[37]
Taenia crassiceps	Colitis-associated colorectal cancer	Decrease recruitment of inflammatory monocytes and inflammation in colon	[27]
Toxoplasma gondii	Melanoma	Activation of $CD8^+$, NK cells, and expression of MHC-I and MHC-II in APC	[12]
	Fibrosarcoma	Increase in the activity of cytotoxic T cells	[13]
	Melanoma and lung cancer	Suppression of neovascularization via induction of hypoxia and avascular necrosis	[31,32]
Trichinella spiralis	Melanoma	Reduction of lung metastasis through CXCL9, CXCL10, IL-4, CXCL1 and CXCL13	[30]
	Human hepatoma cell line (HT402) and human chronic myeloid leukemia cell line (K562)	Arrested of the cell cycle in G1 or S phase	[38,39]
Trypanosoma cruzi	Breast and colon cancer	Activation of $CD4^+$ and $CD8^+$ cells and production of antibodies against cancer cells	[8]
	Experimental breast adenocarcinoma	*Trypanosoma cruzi* calreticulin as a revealer of the presence of tumor cells in the immune system	[9]
	Mammary cancer	Inhibition of proliferation and migration of endothelial cells	[33]
	Melanoma	J18 recombinant protein induces apoptosis through caspase 3	[36]

The possible mechanisms of action of these parasites on blocking the development of different neoplasms has been organized with the purpose of summarizing the advances that have been made over the last years of research in immunotherapy with biological agents in cancer.

AOM/DSS model and with the knowledge provided by previous studies that state that the larval stage of *Taenia crassiceps* (larval stage that grows only in the peritoneal cavity) down-regulated the inflammation and improved the outcome of acute DSS-induced colitis [26], it was tested whether the previous extraintestinal infection caused by this helminth was able to inhibit the development of carcinogenesis in colon associated with inflammation. Interestingly, *T. crassiceps* preinfection reduced significantly (60%) the total number of tumors in the colon compared with uninfected mice, whereas 50% of the mice infected with this cestode did not develop tumors [27]. Such remarkable effect was associated with down-modulated recruitment of inflammatory monocytes and inhibition of local exacerbated inflammatory responses in the colon. In addition, this infection promoted alternative activated macrophages (M2) polarization and down-regulated the IL-17 production, as well as a reduction on the expression of several tumor markers such as β-catenin and COX-2 together with a Ki67 expression reduction, which is associated with cell proliferation. However, the specific antitumor mechanisms induced by the *T. crassiceps* infection during CAC development are still unknown, but some possible targets are suggested in Table 1. Currently, it has been observed that products released by the larval stage of *T. crassiceps* also regulate the development of CAC targeting on several intracellular pathways associated with tumorigenesis (personal observation). In contrast, an intestinal helminth infection caused by *Heligmosomoides polygyrus*, in the early stages of CAC promoted both inflammation and tumorigenesis in colon through the reduction of $CD8^+$ effector T cells [28]. These contrasting results may arise from some differences in the parasitic models used, whereas *T. crassiceps* infection remains in the peritoneal cavity of their hosts and its chronic, *H. polygyrus* causes an intestinal infection with a limited life-span in this tissue. Moreover, in this last model, the DSS treatment could have damaged the epithelium in the small intestine and promoted an accelerated expulsion of the worm, thus avoiding a potential regulatory effect of *H. polygyrus* on tumorigenesis in the colon. Whereas *T. crassiceps* infection appears to positively modulate antitumor responses during CAC, there are reports with clear contrasting effects of other helminthic infections that seems to promote carcinogenesis such as those found in schistosomiasis and liver fluke, which were shown to be associated with bladder cancer and cholangiocarcinoma, respectively [29]. Therefore, more specific and more comprehensive studies are necessary to clarify the potential beneficial and/or harmful effects caused by helminthic diseases in different types of tumors.

Targeting hallmark: activating invasion and metastasis

The process of metastasis is a feature of carcinomas with a higher pathological degree of malignancy, in which the cancer cells acquire the ability to spread from the primary tumor to distant tissues. The first step is the invasion toward local tissues, then the intravasation of cancer cells to lymphatic tissue and nearby blood vessels, followed by the transport of these cells to distant tissues and their extravasation. The last step is to generate small nodules of cancer cells and gradually colonize new tissues. One of the many characteristics is that a cancer cell develops its ability to stimulate the production of chemokine ligands that promote its invasive behavior [2]. The effect of *Trichinella spiralis* on a melanoma model based on a subcutaneous injection of B16-F10 cells has been described within the immunotherapy with biological agents, where the previous oral infection with L1 larva of *T. spiralis* decreased tumor growth and its metastasis to the lungs by reducing the production of some chemokines, such as CXCL9, CXCL10, CXCL1, CXCL13, and IL-4 [30]. Interestingly, the increase of CXCL10 has also been associated with advanced human cancers such as malignant melanoma. In the present report, the group that was infected with *T. spiralis* and challenged with a melanoma cell line showed a greater reduction of CXCL10 production in comparison with the other group that was only infected with *T. spiralis*. This could imply that the immune profile generated by the infection could alter the response and change it into another antigenic stimulus, and such modification could have a regulatory effect on tumorigenesis, at least regarding this type of neoplasm.

Targeting hallmark: inducing angiogenesis

The neovascularization associated with a tumor is generated by the process of angiogenesis, which is essential to supply nutrients and oxygen to the tumor cells for its neoplastic growth. It has been reported that protozoan infections are key in this process, thus during the acute phase of infection with *T. gondii*, there is an increase in the production of type II IFNs and cytokines that possess antiangiogenic properties. In an *in vivo* model of melanoma and in a Lewis lung cancer model, a *T. gondii* infection inhibited neoplastic growth through suppression of neovascularization via induction of hypoxia and avascular necrosis [31,32]. In addition, not only a liver infection may alter vascularization in a tumor, but also parasite-derived molecules may have an impact. For example, exposure to calreticulin derived from *T. cruzi*, in addition to promoting an antitumor immune response as mentioned above, may induce an antiangiogenic effect in breast tumors, both *in vitro* and *in vivo*, where *T. cruzi* calreticulin was able to inhibit the migration and proliferation of endothelial cells, possibly due to the internalization of this protein in the epithelial cells [33].

Targeting hallmark: resisting cell death

Apoptosis is a physiological process of cell death triggered by intracellular signals or extracellular environment, which plays a critical role in the development and homeostasis of normal tissue. During the development of cancer, apoptosis functions as a barrier to contain the excessive proliferation of transformed cells. However, it can be attenuated in tumors with a higher degree of transformation and resistance to therapies [2]. Protozoa and helminths have been described as inducers of apoptosis, which is a survival mechanism, in cells of the immune system and epithelial cells [34,35]. This proapoptotic effect has also been tested in both *in vivo* and *in vitro* cancer models. The first signs of the antitumor effect of a parasite were observed in patients with chronic infections caused by *T. cruzi*. As it was previously mentioned, despite having the conditions for this tumorigenic process, epidemiological data indicate the absence of colon cancer in patients with chagasic megacolon [7]. Chronic inflammation caused by this pathology can lead to the development of mutations in epithelial cells or to the maintenance of these preneoplastic and neoplastic cells, which could suggest an important correlation between *T. cruzi* infection and a lack of development of colorectal cancer. Not only has the antitumor effect of *T. cruzi* infection been reported through the activation of the immune system, but also through a putative proapoptotic activity of the components of this protozoan. Some specific compounds from *T. cruzi* have shown a proapoptotic *in vitro* activity on several cell lines. For instance, the recombinant J18 protein based on gp82, a surface molecule of *T. cruzi*, induced apoptosis on melanoma cells without affecting the normal melanocytes. Furthermore, *in vivo* inoculation of recombinant J18 together with tumor cells induced tumors of smaller size. Other proapoptotic compounds detected in this parasite, such as Tc52, have been shown to down-modulate cell survival on different tumor cell lines, and this effect was associated with an increase in the activity of caspase 3 [36]. Therefore, such *T. cruzi*-derived molecules may reduce apoptosis-resistance in melanoma cell lines. This and other mechanisms of action of the *T. cruzi* components have not been fully described yet. For example, it is unknown whether these compounds are able to bind to some kind of receptor on the surface of the transformed cells or whether such compounds can directly cross the cell membrane and activate proapoptotic signals.

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Targeting hallmark: sustained proliferation signaling

Sustained chronic proliferation is a skill possessed by cancer cells through the deregulation of mitogenic signals. In an *in vitro* model of fibrosarcoma cells, treatment with protoscolices of hydatid cysts inhibited the proliferation of cancer cells [37]. Also, the crude extract of *Trichinella spiralis* inhibited the cell proliferation through the arrest of the cell cycle in the G1 or S phase of the human chronic myeloid leukemia cell line K562 and the hepatoma cell line H7402 [38,39]. The involved mechanisms are not clear yet, although during the formation of the *T. spiralis* cysts the protein p53 is expressed [40], which has the function of regulating the cell cycle, which may lead to the inhibition of tumor growth.

Concluding remarks and future directions

In this review, we have gathered information about the different antitumor mechanisms triggered by some helminth and protozoan parasites, together they may target ∼50% of the hallmarks of cancer. In spite of having parasites that are classified as inducers and promoters of some neoplasms, others are reported as negative regulators of cancer. As described above, parasites can interfere in the growth and proliferation of a variety of transformed cell lines *in vitro*, but also, and more importantly, parasites and their products can modulate cancer development *in vivo* from melanoma to colon cancer. However, the mechanisms of action triggered by the parasites and some of their products involved in modulating cancer development are diverse and even not yet fully described. Their variability of antitumor response depends on several factors: the type of cancer and even the stage of transformation in which it is found, as well as the immune response generated by the host against the infection in progress, thus not all parasites and their molecules may have the same effect on carcinogenesis. Whereas some of them can activate the immune response in a by-standing way, such as unspecific activation of immune cells or inducing cytokines or reducing chemokines, other parasites can affect the cell cycle and stop cell proliferation in transformed cells (cell arrest) and generate a chance for the action of toxic drugs, thus promoting cancer elimination.

It is important to mention that the infections caused by helminths and protozoa to eliminate cancer could result in an impractical idea, given that such intervention may cause some harm to the host or unexpected infections by using alive parasites. However, this inconvenience may be outweighed by the identification of the products they secrete, as well as the molecules associated with their composition, with a direct effect on cancer cells or on the tumor microenvironment. This may be the key to create new antitumor treatments. The use of parasitic infections to modulate or impact positively on different inflammatory diseases and cancer should not be done with the aim of substituting the drugs in current use, but as a probe of concept for the potential use of their derivative molecules. We do not propose replacing the drug treatment with parasites or their molecules, but we rather propose to conduct more comprehensive studies on such potential molecules as regulators on cancer. Therefore, parasite biological factors may be used in a future as adjuvants to improve the effect of the current drugs. Although several *in vitro* studies have demonstrated that some molecules derived from parasites can induce apoptosis, only one study has been linked to the specific modulation of caspase 3 [36] and the remaining experiments have not defined the putative targets of such parasite molecules. Thus, it is mandatory to define more specific pathways affected by such treatments. For example, a common *in vitro* report states that exposure of cell lines or lymphoid cells to some parasitic extracts or semipurified molecules supports the idea of inhibition of cell proliferation [41]; therefore, it is essential to analyze the phases of the cell cycle that are being affected, such as the progression or arrest between the cell cycle G1, S, G2 and M phase, as well as to analyze the involved cyclins that may be down or up-regulated. Moreover, at least three different helminth infections have demonstrated to modulate the JAK–STAT signaling pathway [42] and to decrease, under certain circumstances, the NF-kB activity [43,44]. It is worth to note that such intracellular signaling pathways are consistently found altered in different types of cancer cells [45]. Thus, more detailed studies on the effects of helminths/protozoa and their products on these signaling pathways are crucial. In line with this idea, it has been reported that some helminth infections contribute to the overexpression of SOCS 1 and SOCS 3 [46,47], which blocks the JAK-STAT signaling, whereas other parasitic diseases have been found to inhibit STAT1 activation in response to IFN-γ [46]. Even such parasite-derived molecules may serve as 'distractors' to avoid the recruitment of harmful inflammatory cells in the tumor microenvironment. Additionally, a key research that must be conducted in the next few years is the search for putative receptors for parasitic-derived molecules in the immune cells or in the affected tissue, such as epithelial cells, as well as the signaling pathways that they may turn on or off in such cells.

Thus, the use of molecules derived from biological agents together with chemo and immunotherapy could be another key element to promote treatments directed against cancer. This is why, it is essential to have a deeper understanding regarding their potential antitumor activity.

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

AOM, azoxymethane; APC, antigen-presenting cells; CAC, colitis-associated colorectal cancer; DSS, Dextran Sodium Sulfate.

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