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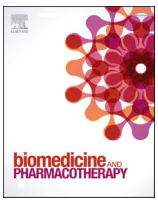

Helminth-derived molecules improve 5-fluorouracil treatment on experimental colon tumorigenesis

Mónica G. Mendoza-Rodríguez [a,*], Daniela Medina-Reyes [a], Cuauhtémoc A. Sánchez-Barrera [a], Karen V. Fernández-Muñoz [a,b], Verónica García-Castillo [a], Jorge L. Ledesma-Torres [a], Marisol I. González-González [a], José L. Reyes [a], Carlos Pérez-Plascencia [a,c], Miriam Rodríguez-Sosa [a], Felipe Vaca-Paniagua [a,d], Marco A. Meraz [b], Luis I. Terrazas [a,d,**]

[a] *Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Avenida de los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, Mexico*

[b] *Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Avenida Instituto Politécnico Nacional 2508, Ciudad de México 07360, Mexico*

[c] *Laboratorio de Genómica, Instituto Nacional de Cancerología, Tlalpan, Mexico*

[d] *Laboratorio Nacional en Salud, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de Mexico, Avenida de los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, Mexico*

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ABSTRACT

Colorectal cancer (CRC) is one of the most prevalent fatal neoplasias worldwide. Despite efforts to improve the early diagnosis of CRC, the mortality rate of patients is still nearly 50%. The primary treatment strategy for CRC is surgery, which may be accompanied by chemotherapy and radiotherapy. The conventional and first-line chemotherapeutic agent utilized is 5-fluorouracil (5FU). However, it has low efficiency. Combination treatment with leucovorin and oxaliplatin or irinotecan improves the effectiveness of 5FU therapy. Unfortunately, most patients develop drug resistance, leading to disease progression. Here, we evaluated the effect of a potential alternative adjuvant treatment for 5FU, helminth-derived Taenia crassiceps (TcES) molecules, on treating advanced colitis-associated colon cancer. The use of TcES enhanced the effects of 5FU on established colonic tumors by downregulating the expression of the immunoregulatory cytokines, *Il-10* and *Tgf-β*, and proinflammatory cytokines, *Tnf-α* and *Il-17a*, and reducing the levels of molecular markers associated with malignancy, cyclin D1, and Ki67, both involved in apoptosis inhibition and the signaling pathway of β-catenin. TcES+5FU therapy promoted NK cell recruitment and the release of Granzyme B1 at the tumor site, consequently inducing tumor cell death. Additionally, it restored P53 activity which relates to decreased *Mdm2* expression. In vitro assays with human colon cancer cell lines showed that therapy with TcES+5FU significantly reduced cell proliferation and migration by modulating the P53 and P21 signaling pathways. Our findings demonstrate, for the first time in vivo, that helminth-derived excreted/secreted products may potentiate the effect of 5FU on established colon tumors.

1. Introduction

Colorectal cancer (CRC) is one of the world's most prevalent and deadly neoplasias. In 2020, CRC was the third most common cancer in men and the second most common in women. The mortality rate of CRC is expected to increase within the next decade, mainly affecting individuals in highly developed countries [1]. The pathogenesis of CRC is complex, but it is well known that approximately 75% of CRC cases are sporadic. Poor dietary habits, smoking, obesity, and low physical activity are risk factors [2]. CRC development occurs via a multistep process that accumulates genetic and morphological changes over 10–15 years [3]. Some reports indicate the importance of early detection

* Corresponding author.
** Corresponding author at: Unidad de Biomedicina, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Avenida de los Barrios 1, Los Reyes Iztacala, Tlalnepantla 54090, Mexico.
E-mail addresses: monica.mendoza@iztacala.unam.mx (M.G. Mendoza-Rodríguez), literrazas@unam.mx (L.I. Terrazas).

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and early care of CRC patients to decrease mortality rates. Unfortunately, nearly a quarter of patients are diagnosed in the advanced stage with tumor invasion to other organs, and it is estimated that approximately 50% of patients diagnosed in the early stage will develop metastasis in five years, which contributes to the high mortality rates of CRC [4]. The first-line treatments in stages III and IV are surgery and chemotherapeutics. 5-Fluorouracil (5FU) remains the axis chemotherapeutic agent for colorectal cancer treatment in early and metastatic stages, even though it has a limited response rate as a single-agent treatment (10–15%). Combination treatment with other chemotherapeutics agents, such as oxaliplatin or irinotecan, has been applied to increase its effectiveness [5]. Nevertheless, acquired or intrinsic drug resistance significantly reduces the efficacy of conventional treatments, posing substantial clinical challenges. Thus, searching for new therapeutic strategies that enhance the patient's response to current chemotherapeutics is necessary.

The impact of different pathogens on cancer development has been broadly reported since it was found that human papillomavirus and Helicobacter pylori directly participate in the progression of cervix and stomach cancers, respectively. Moreover, other pathogens can modulate the development of different types of cancer. Highly industrialized countries present a high incidence of CRC but a low incidence of helminthic infections; thus, an inverse relationship may exist. Chronic inflammation is a hallmark of cancer development, and CRC is closely related to chronic inflammatory processes. Helminth parasites have been recognized to modulate host immunity, mainly by suppressing inflammatory responses. However, whether helminths are facilitators or delayers in the onset of colon cancer has yet to be elucidated [6].

Our previous data have supported that a preexisting extraintestinal helminth infection caused by Taenia crassiceps may delay the development of colitis-associated colon cancer (CAC) [7]. Later, we used excreted/secreted products (TcES) from this cestode and found that they similarly delay CAC development by downregulating pro-inflammatory and pro-tumorigenic signaling pathways, suggesting that an entire helminth infection is unnecessary to improve CAC outcomes [8]. More recently, new data from different authors supported our original findings, indicating that helminths and their derivatives may help modulate the development of certain kinds of cancer or the growth of cancerous cells [9–11].

Here, we demonstrated for the first time that TcES is potentially helpful as an adjuvant to significantly improve the effectiveness of 5FU by modulating inflammatory cytokines, altering the tumor microenvironment, downregulating markers associated with malignancy, and promoting the release of granzyme B1 (GrB1), apoptosis, and P53 expression levels to reduce colonic tumor growth in advanced CAC.

2. Materials and methods

2.1. Mice

Female BALB/c mice, eight to ten weeks old, were purchased from UPEAL Laboratories, CINVESTAV (México), and maintained in a specific 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 had access to water ad libitum.

2.2. Molecules excreted/secreted by Taenia crassiceps

Parasite harvest and infection were performed as previously described to obtain the molecules excreted/secreted by *T. crassiceps* [8]. Briefly, the metacestodes of *T. crassiceps* (ORF strain) were harvested from the peritoneal cavity of female BALB/c mice under sterile conditions after eight weeks of infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS) and cultured in PBS at 37 °C for 24 hr. The supernatant was recovered and centrifuged for 10 min at 5000 rpm, and the proteins were concentrated using an Amicon Ultrafilter with a 50-kDa cutoff membrane (Millipore, Billerica, MA, USA).

2.2.1. Macrophage culture and nitric oxide level measurement

As previously described, single-cell suspensions of bone marrow-derived macrophages isolated from the femurs and tibias of naïve mice were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% Fetal bovine serum, penicillin, and streptomycin and cultured at with 5% CO2. Macrophage colony-stimulating factor (10 ng/ml; R&D Systems, UK) was added to the bone marrow cultures on days 0, 2, and 4. On day 7, the cells were exposed to 25 μg of TcES for 5 min previously at the stimulus with Lipopolysaccharides (LPS) (1 μg/ml), both maintained until harvest. Supernatants were collected 24 h later, and the nitric oxide (NO) concentration was determined using Griess reagent according to the manufacturer's instructions.

2.3. Induction of colitis-associated colorectal cancer

The CAC model was established as previously described. Briefly, BALB/c mice received an intraperitoneal (i.p.) injection containing 12.5 mg/kg azoxymethane (AOM; Sigma Aldrich, St. Louis, MO, USA). Five days later, 2% dextran sodium sulfate (DSS, MW: 35,000–50,000, MP Biomedicals. Irvine, CA, USA) was dissolved in the animals' drinking water for seven days. Then, the mice received regular drinking water for 14 days. This experimental series was repeated twice. Throughout the experiment, the mice were monitored weekly for body weight, stool consistency, and blood in the rectum or stools.

2.3.1. Fluorouracil and T. crassiceps-derived molecules treatment in the mouse CAC model

On day 54 after AOM injection, the CAC-induced mice were divided into four groups; two groups began treatment with molecules excreted/secreted by *T. crassiceps* (TcES) at concentrations indicated in a previous report [8]. Briefly, 200 μg of TcES products were intraperitoneally inoculated into each mouse three times weekly. Seven days later, a group of mice initially receiving TcES treatment received additional (co-treatment) i.p. injections of 30 mg/kg 5FU (TcES+5FU). At the same time, the previously untreated CAC mice received 5FU as a single therapy (5FU). CAC-induced mice without treatment were utilized as a control group (CAC). All the mice were euthanized on day 90 after AOM injection, and healthy mice were included as a negative control (Ctrl). The colons were removed, weighed, and processed for macroscopic inspection and histopathological examination. Immediately, the colonic tissue was either fixed in 100% ethanol and embedded in paraffin for histopathologic analysis or snap-frozen in liquid nitrogen for RNA and protein extraction.

2.4. Histologic analysis

Tissues were collected from mice in different CAC treatment groups (5FU, TcES, and TcES+5FU) and an untreated CAC group (CAC). Finally, the tissue obtained from healthy mice (Ctrl) was fixed in 100% ethanol and embedded in paraffin for posterior 4-mm cross-sectioning. The tissue sections were stained with hematoxylin and eosin (H&E) for pathologic evaluation or with Alcian blue stain to visualize the goblet cells using an optical Leica SP8. These cells were quantified as a percentage from at least 20 crypts per region in five fields per slide. Four different slides per mouse were analyzed at 10X and 40X magnification.

For immunohistochemical and immunofluorescence staining, sections were incubated overnight for analysis; the sections were deparaffinized in xylene and then rehydrated with a series of graded alcohols and processed as previously reported [8]. The sections were incubated overnight at 4 °C with the following primary antibodies diluted in 1X PBS: anti-E-cadherin, 1:300 (Cell Signaling Technology, Danvers, MA);

anti-β-catenin, 1:200 (GeneTex, Irvine, CA, USA) and anti-CD335 (Thermo Fisher, Waltham, MA, USA). The antibodies utilized for immunofluorescence were anti-cyclin D1 (Cell Signaling Technology, Danvers, MA, USA), Ki67, and GrB1 (all from GeneTex, Irvine, CA). All tissues were developed following the conventional technique. The slides were analyzed using confocal microscopy analysis performed using a Leica TCS SP8 confocal microscope system. Immunohistochemistry and immunofluorescence quantification were performed by counting cells in 10 high-powered fields from each mouse using ImageJ software v.4.9.

2.5. RNA extraction and gene expression

Total RNA was extracted from colon tissue using the DNA/RNA/PROTEIN Kit purification plus (NORGEN BioTek Corp., Thorold, ON, Canada) following the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260 nm. One microgram of RNA was used for first-strand cDNA synthesis with a Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was carried out using the Fast Start SYBR Green Master kit (Applied Biosystems, Foster City, CA, USA) using a CFX96 C1000 Thermal Cycler (Bio-Rad). Relative gene expression values were normalized to the constitutive expression of the β-Actin gene. Specific primers for target genes are shown in Supplementary Table S1.

2.6. Flow cytometry

For flow cytometry, peritoneal exudate cells were obtained during animal necropsy. 1×10^6 cells were resuspended in cold PBS containing 0.5% FBS and 1 mM EDTA (FACS buffer). Fc receptors were blocked with anti-CD16/32 antibodies. The cells stained were made with fluorochrome-conjugated antibodies against different cell surface markers, including CD3 (BD Biosciences), CD4, CD8, and CD49b (BioLegend). Finally, 100 μl of each sample were analyzed on the Attune NxT flow cytometer (ThermoFisher®, Rockford, IL, USA), data analysis was performed with FlowJo Software V.10.8.1.

2.7. Fluorescent TUNEL assay

The death of tumor cells was assessed by fluorescent labeling of terminal dUTP nick end labeling (TUNEL) using an In Situ Cell Death Detection Fluorescein Kit (Roche, Basilea, Swiss) following the manufacturer's instructions. The samples were analyzed with a ZeissVert A1 conventional epifluorescence microscope and a LEICA TCS SP2 confocal microscope. The analyzed area in each sample was 2.8 mm2, and 20 fields of 50 mm2 were evaluated.

2.8. SDS—PAGE and western blotting

Total proteins were obtained from tissue lysates with the RNA/Protein purification Kit (NorBiotek Canada, Thorold, ON, Canada). Proteins were separated by 12% SDS—PAGE, blotted onto nitrocellulose membranes, and blocked. The membranes were then exposed to mouse anti-P53 (1:1000) or anti-P21 (1:1000) (Cell Signaling Technology, Danvers, MA, USA) and anti-YB1 antibodies (from AB Clonal, Woburn, MA, USA). The anti-β-actin monoclonal antibody (Santa Cruz Biotechnology, St. Cruz, CA, USA) was utilized to detect cell actin as the protein load control. Horseradish peroxidase (HRP)-tagged secondary anti-rabbit or anti-mouse antibodies (1:5000) (Jackson ImmunoResearch, West Grove, PA, USA) were used for chemiluminescent detection with the ImmobilonTM Western Chemiluminescent HRP Substrate kit (Millipore, Burlington, MA, USA). Chemiluminescence signals were recorded on an Alliance Q9 UVITEC imaging device (Cambridge, UK), and densitometric analyses were performed with ImageJ software.

2.9. Cell line culture and treatment

The HT-116 and RKO cell lines (ATCC, Manassas, VA, USA) were cultured in McCoy's 5 A medium (Gibco, Grand Island, NY, USA) and Minimum Essential Medium (MEM) medium, respectively. All cultures were supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin and incubated at 37 °C with 5% CO2. A total of 2.5×10^3 cells/cm^2 were plated and cultured for 24 h for experiments. After switching to McCoy's 5 A and MEM medium, respectively, with 1% Fetal bovine serum (FBS) for 18 h, the cells were cultured without serum and stimulated with 50 μg of TcES for 24 h. Next, 10 μM 5FU (Sigma Aldrich, USA) for 24 h was added to the cells previously stimulated with TcES. At the same time, cells stimulated with TcES or 5FU alone were cultured. Control cells (without treatments) were also cultured.

2.9.1. Migration assay

A wound healing assay was performed to evaluate cell migration as previously described [12]. Briefly, HCT-116 and RKO cell lines were seeded at a concentration of 4×10^5 cells/well in 6-well plates with 10% FBS-supplemented medium at 37 °C for 24 h and then switched to medium with only 1% FBS to avoid cell proliferation for 24 h. A wound scratch was performed with a sterile 200 μl pipette tip in each well. Then, the medium was removed and replaced with fresh medium under the same conditions. Images of wounds in the cell culture were captured every 24 h for 72 h using a ZeissVert A1 conventional epifluorescence microscope. The wound closure areas were measured with ImageJ (Software 1.48q, Rayne Rasband, National Institutes of Health, USA).

2.10. Statistical analysis

Data were analyzed either by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests or by unpaired two-tailed t-tests with GraphPad Prism 5 (San Diego, CA, USA). All tests were performed using 95% confidence intervals. Data are expressed as means ± standard deviation (SD), where * represents $p < 0.05$ and ** represents $p < 0.01$.

3. Results

3.1. Exposure to excreted/secreted products from Taenia crassiceps (TcES) increases the antitumorigenic effect of 5-fluorouracil on advanced colitis-associated colon cancer

Here, we evaluated the role of TcES in well-established colon tumors and its possible beneficial effect as an enhancer of 5FU therapy in advanced CAC. Before being used in vivo, TcES was verified to be endotoxin-free (≤0.015 EU/ml) and biologically functional through its ability to inhibit NO production in LPS-stimulated macrophages (Fig. 1A). LPS-stimulated macrophage exhibited increased NO levels, as expected. However, LPS-stimulated macrophages exposed to TcES had significantly reduced NO levels (~70%). After confirmation of the biological activity of TcES, it was administered as indicated in Materials and Methods. Treatments with 5FU or TcES alone or in combination (TcES plus 5FU) were individually analyzed in the mouse AOM/DSS CAC model (Fig. 1B).

As shown in Figs. 1C and 1D, we found that CAC-untreated mice (CAC) developed more than 20 colonic tumors in the distal and middle regions of the colon (Fig. 1C). In contrast, mice receiving monotherapy with 5FU from day 61 until day 90 (5FU group) displayed a slight but nonsignificant reduction of ~15% in the number of colon tumors (Fig. 1D). Mice treated with TcES as monotherapy developed 40% fewer tumors than untreated CAC mice. Remarkably, compared to untreated mice, mice that received 5FU plus TcES as an adjuvant exhibited a significantly reduced number of colonic tumors (~70% less). Thus, compared to 5FU monotherapy, TcES treatment enhanced and improved the effect of 5FU by approximately 50% (Fig. 1D).

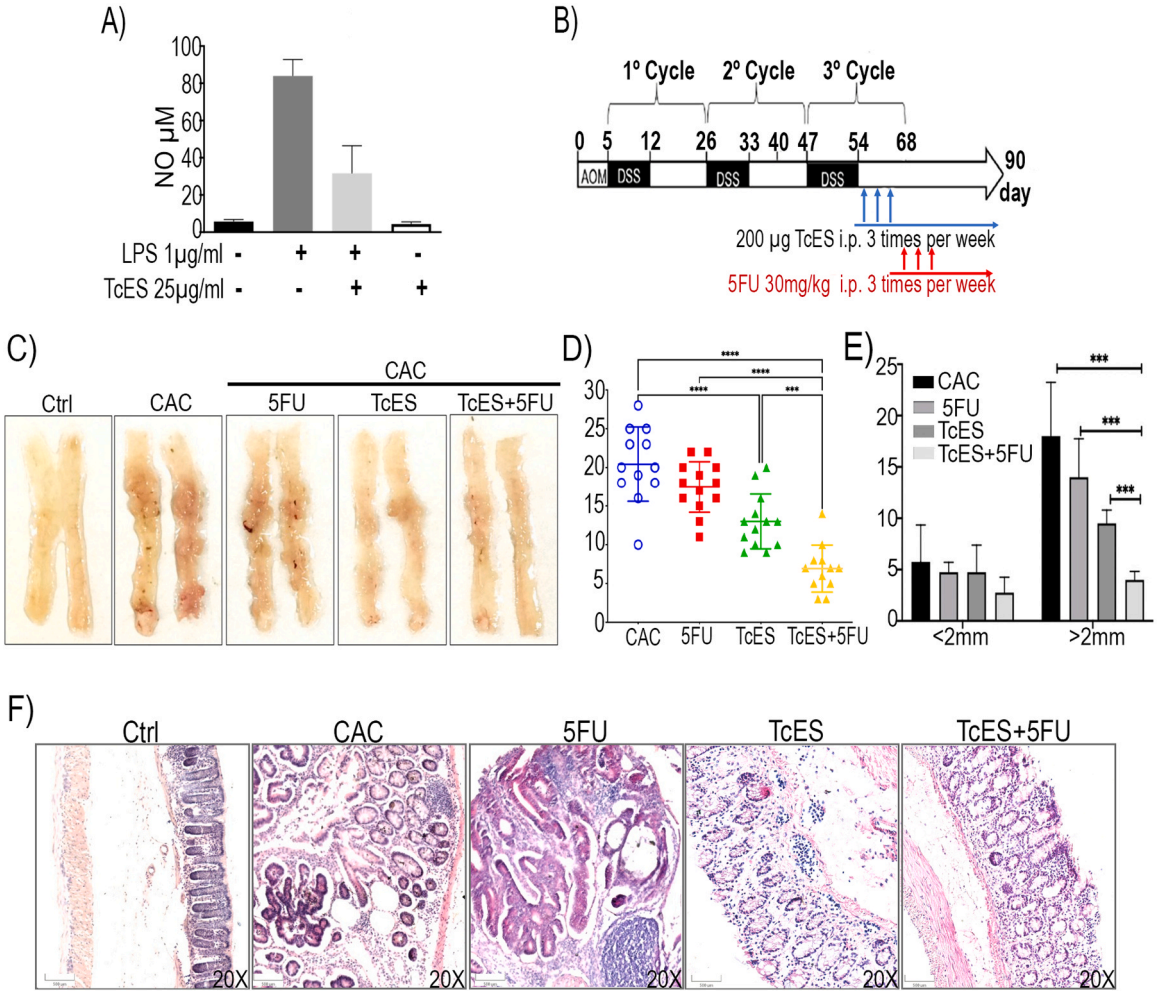

Fig. 1. Molecules excreted/secreted by *Taenia crassiceps* (TcES) promote the antitumor effect of 5FU on colorectal cancer. (A) Nitric oxide (NO) production in the supernatants (24 h) of bone marrow-derived macrophages stimulated and nonstimulated with LPS and exposed or not to TcES was measured by the Griess reaction. (B) Experimental design of CAC induction and administration of treatments. The scheme indicates the days of exposure to azoxymethane (AOM) and dextran sodium sulfate (DSS) and when mice were treated with 5FU and TcES either as a single therapy or in combination. (C) Images of colons dissected longitudinally, showing macroscopic morphology after CAC induction and treatment with or without 5FU, TcES, and combination therapy (TcES+5FU) on day 90. (D and E) Average number and size of tumors in CAC mice without treatment (CAC), 5FU, TcES, and a combination of TcES+5FU. (F) Representative H&E-stained colonic sections from healthy mouse tissue (Ctrl), untreated CAC (CAC), CAC treated with 5FU (5FU), CAC treated with TcES (TcES), as single therapies, and the combined effect of TcES and 5FU (TcES+5FU) on CAC. p value lower than 0.001 (***); p = 0.01 (**) and p = 0.05 (*). Data are representative of three independent experiments. n = 4–6 mice per group.

Additionally, mice treated with TcES+5FU displayed smaller tumors in the colon than those found in untreated CAC mice and mice treated with 5FU monotherapy (Fig. 1D–E). Next, sections of colon tissues were stained with hematoxylin and eosin to determine morphological changes in the tumorigenic sections. In Fig. 1F, sections of healthy colon tissue (Ctrl) showed well-defined crypts lined up in parallel. In contrast, CAC untreated mice (CAC)-tissues and 5FU single therapy tissues showed an altered morphology, displaying glandular adenocarcinomas consisting of atypical epithelial cells with dysplastic nuclei and numerous mitotic figures and chronic inflammation confined to the lamina propria. In mice treated with TcES alone, less severe intestinal inflammatory cell infiltration and crypt distortion were observed compared to those in CAC-untreated mice. The combination treatment of TcES+5FU significantly reduced crypt size and tissue damage; however, some hyperplasia was still observed (Fig. 1F).

3.2. TcES, as an adjuvant to 5FU, restores mucosal homeostasis and downregulates inflammatory cytokines in colon cancer-associated colitis

The alteration in mucus production by goblet cells is considered a hallmark of ulcerative colitis and CRC [13,14]. To demonstrate the possible anti-inflammatory and epithelial protective effects of TcES as a single therapy and in combination with 5FU, we evaluated the number of goblet cells in the tissue of CAC mice under different conditions. As shown in Figs. 2A and 2B, the number of goblet cells was significantly reduced (~90%) in CAC-untreated samples (CAC) versus healthy tissues (Ctrl). This reduction was also observed in the colon of mice treated with 5FU, where a decrease in mucin production and loss of the characteristic crypt architecture was evident. Strikingly, in mice that received TcES alone or in combination with 5FU, the number of goblet cells increased four- and five-fold, respectively, suggesting a possible protective effect of TcES on the integrity of the mucosal barrier (Figs. 2A and 2B). The alteration in the mucosal barrier can lead to a sustained inflammatory condition that favors colon carcinogenesis and resistance to cancer treatment in advanced stages [13,14].

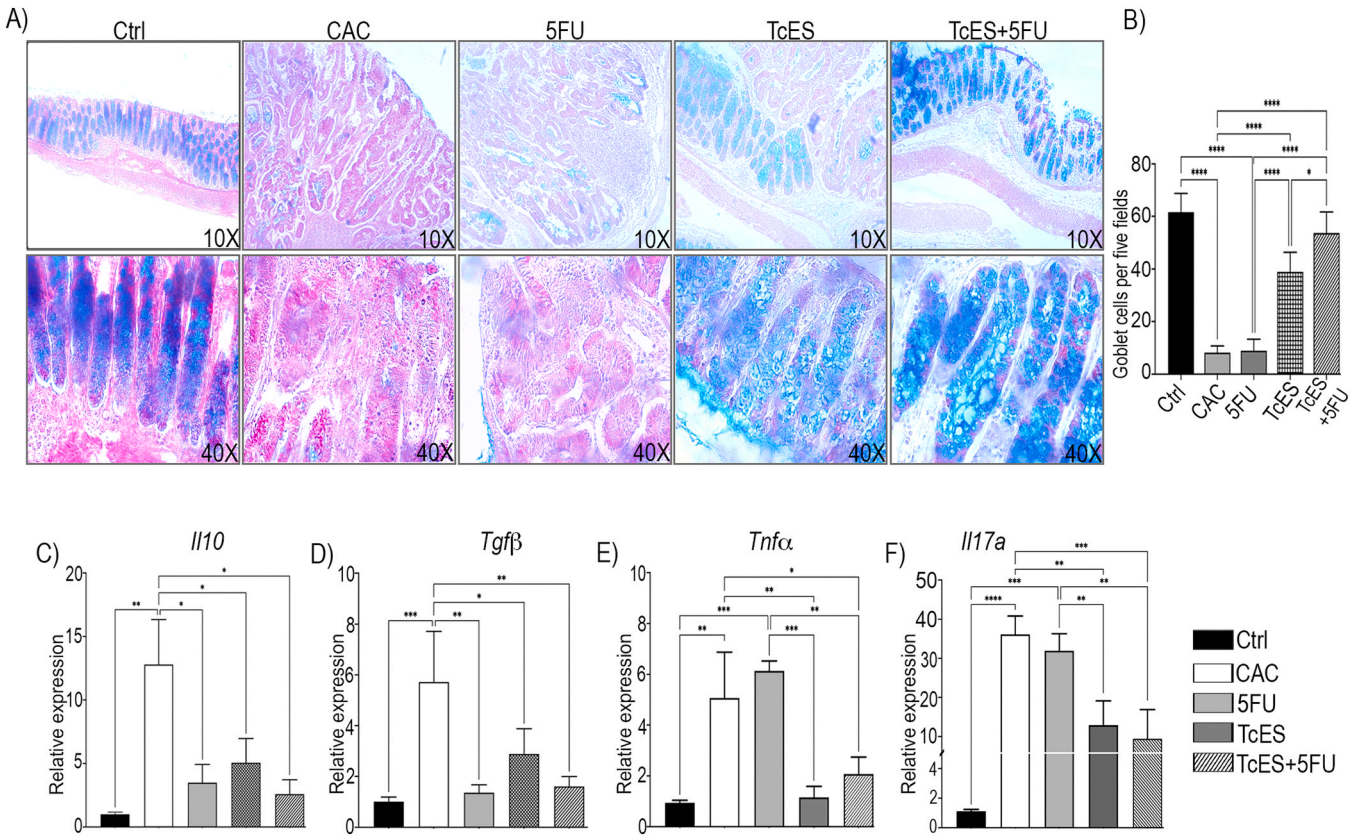

Fig. 2. The adjuvant effect of TcES restores mucosal homeostasis and decreases inflammatory cytokine levels in colon of cancer-associated colitis mice. (A) Representative colon tissues from mice subjected to different treatments (5FU, TcES, and TcES+5FU) and tissues from untreated CAC mice (CAC) and healthy mice (Ctrl) stained with Alcian blue to visualize goblet cells at 90 days after CAC induction. (B) Quantitative representation of goblet cell numbers. (C) Relative expression of IL-10, TGF-β, and IL-17A mRNA in Ctrl (white bars), untreated CAC (black bars), 5FU (solid gray), TcES (gridded bars) and TcES+5FU (bar with lines) cells was determined by qPCR. Data are presented as individual values with a median and range from three independent experiments. P< 0.001 (***); P< 0.01 (**) and p<0.05 (*).

To examine the possible role of TcES molecules in regulating immunoregulatory and pro-inflammatory cytokines, we evaluated the mRNA expression levels of interleukin-10 (*Il-10*), Transforming growth factor-β (*Tgf-β*), Tumor Necrosis Factor-alpha (*Tnf-a*), and Interleukin 17a (*Il-17a*) in colon tissues by Quantitative Polymerase Chain Reaction (qPCR) analysis. A significant increase (13–126-fold, 5.8-fold, 5-fold, and 37-fold, respectively) in the levels of all mentioned cytokines was found in the colon tissues of untreated CAC-mice (CAC) relative to healthy control mice (Fig. 2 C-F). CAC mice receiving TcES as a single therapy displayed a significant reduction (2-fold) in *Il-10* and *Tgf-β* levels and *Tnf-a* and *Il-17a* mRNA levels in the colon compared to those in the untreated CAC group (Fig. 2 C-F). In contrast, CAC mice treated with 5FU exhibited downregulated *Il-10* and *Tgf-β* expression (Fig. 2 C-D), but the expression of the inflammatory cytokines *Tnf-a* and *Il-17a* was not modified (Fig. 2 E-F). However, the combination therapy of TcES+5FU significantly decreased the levels of all these factors. Moreover, the mRNA levels of the inflammatory cytokines *Tnf-a* and *Il-17a* were drastically inhibited (3.5-fold and 23-fold, respectively, Fig. 2 E-F).

3.3. TcES reduces the levels of biological markers associated with malignancy by suppressing β-catenin nuclear translocation

The downregulation of E-cadherin expression promotes the loss of the E-cadherin/β-catenin complex, which maintains epithelial cell-cell contact and prevents β-catenin accumulation in the cytoplasm and its nuclear translocation, which could lead to the transcription of genes associated with malignancy and drug resistance [15,16]. Here, we evaluated the protein expression of E-cadherin and β-catenin in the colon tissues of mice with CAC and receiving the treatments mentioned earlier. E-cadherin was strongly expressed in the normal colonic epithelium (Ctrl); in contrast, CAC development in untreated mice significantly reduced its expression nearly to 70%, while mice treated with 5FU as monotherapy did not show any improvement (Fig. 3 A-B). Whereas the colon tissues of mice treated with TcES showed an apparent recovery in E-cadherin expression to the same levels as those in the control group (Fig. 3B). However, compared to 5FU monotherapy, the use of TcES as an adjuvant for 5FU resulted in a 50% upregulation of E-cadherin expression on adherent junctions in the colon (Fig. 3 A-B). The loss of E-cadherin expression is closely related to the accumulation of cytoplasmic β-catenin [17]. In our study, the colon tissues of mice treated with 5FU and CAC mice without treatment displayed intense β-catenin immunostaining mainly in the nuclei (Fig. 3 C), which correlated with downregulated expression of E-cadherin under the same conditions. In contrast, in the colon tissues of mice treated with TcES and TcES plus 5FU, the immunostaining for β-catenin was limited to the cytoplasm and adherens junctions. The results displayed a 50% and 70% reduction in its expression in the cytoplasm and adherens junctions, respectively (Figs. 3A and 3C). In this context, we determined the levels of a specific protein regulated by β-catenin nuclear translocation, Cyclin D1, an oncogene contributing to tumor malignancy and chemoresistance in diverse tumors, including colorectal cancer [16,18]. To evaluate the activation of cyclin D1 and its regulation by TcES in combination therapy with 5FU, colon tissues from the different groups of mice were processed for immunofluorescence labeling of cyclin D1. Colon tissue from CAC mice, as well as from 5FU-treated mice, displayed high nuclear expression levels of cyclin D1. In contrast, in colon tissues obtained

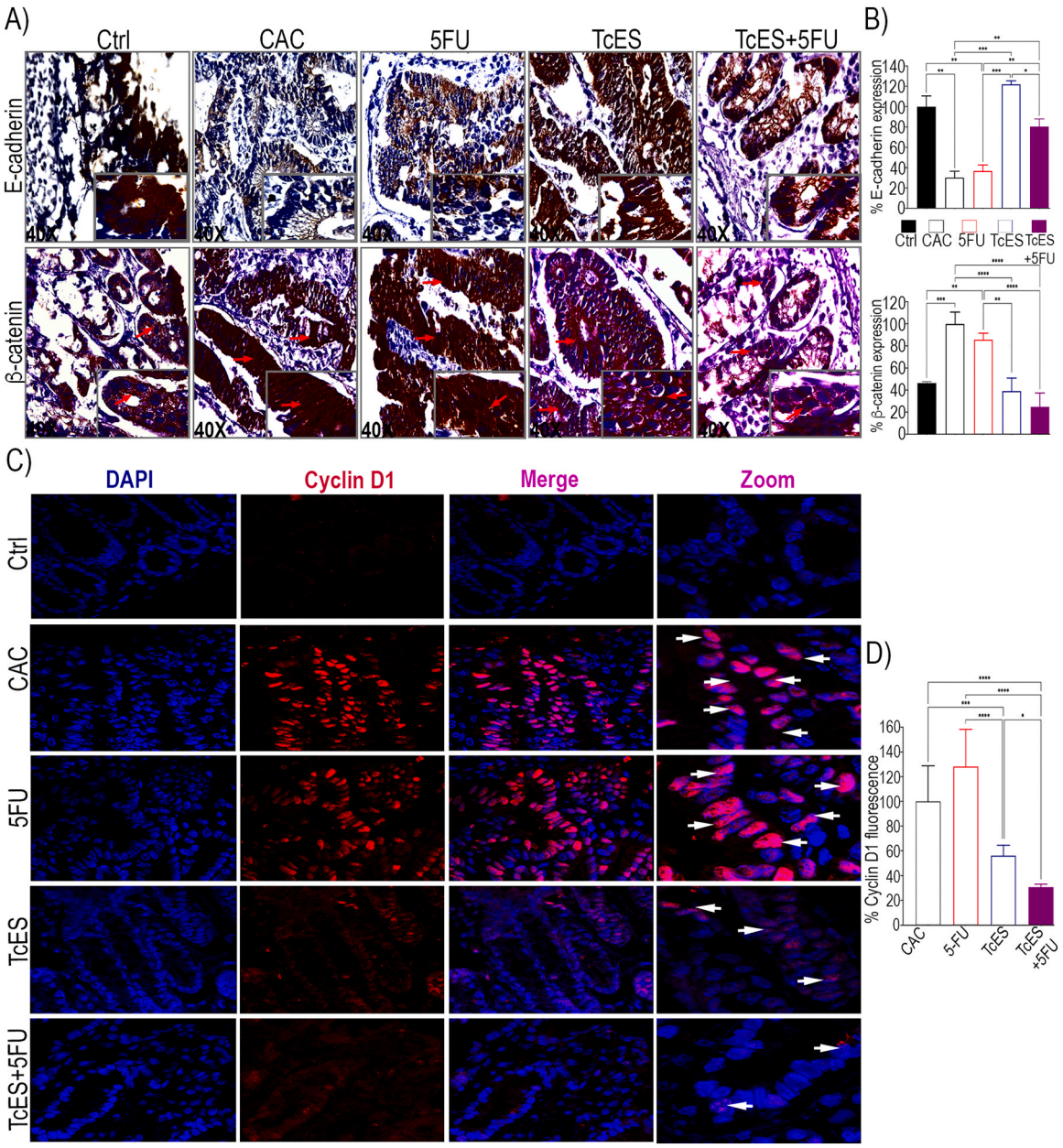

Fig. 3. TcES treatment downregulates epithelial-mesenchymal transition (EMT) markers in mice treated with 5FU. (A and C) Representative immunohistochemical staining of E-cadherin and β-catenin in colon tissue obtained from mice with the previously mentioned treatments. The red arrows show the localization of β-catenin in the epithelial cells. Magnification 40X. (B) Percentage of positive E-cadherin and β-catenin cells. (C) Representative immunofluorescence from colon tissues stained with anti-cyclin D1 antibody and Alexa Fluor 594 to reveal the presence of the protein (red), and DAPI (blue) was used to visualize nuclei counterstaining. Arrows in the merged images point to the nuclear localization of cyclin D1 in tumor cells in CAC and 5FU-treated mice, which was significantly decreased in the colon tissues of TcES-treated mice, while in the nuclei of the tissues from TcES+5FU-treated mice, cyclin D1 was practically abolished; scale bars = 20 μm. (D) Quantitative analysis of the fluorescence of cyclin D1 expression. The tissue sections were analyzed with an SP8 confocal microscope at 40X and 60X. The zoom merge of immunofluorescence was obtained at 60X. Data represent three independent experiments with 4–6 mice per group. The data are expressed as the mean ± SE $P < 0.001$ (***); $P < 0.01$ (**) and $P < 0.05$ (*).

from mice treated with TcES and TcES+5FU, cyclin D1 expression was significantly downregulated by more than 50% and 60%, respectively, compared to that in CAC mice (Figs. 3D and 3E)

3.4. TcES plus 5FU therapy downregulates the expression of the Ki67 proliferation marker

Cyclin D1 expression positively regulates tumor cell proliferation. Thus, we evaluated the effect of TcES on this process through Ki67 expression, a common and broadly employed molecular marker to assess cell proliferation. Additionally, its high expression is associated with metastasis and poor patient prognosis [19,20]. Colon tissues of untreated mice (CAC) displayed upregulated expression of Ki67, while 5FU, as a single therapy, did not modify Ki67 expression (Figs. 4A and 4B). However, treatment with TcES and TcES+5FU significantly reduced the levels of colonic Ki67 immunostaining by ~60% and ~80%, respectively, compared to those in the CAC and 5FU groups (Figs. 4A and 4B).

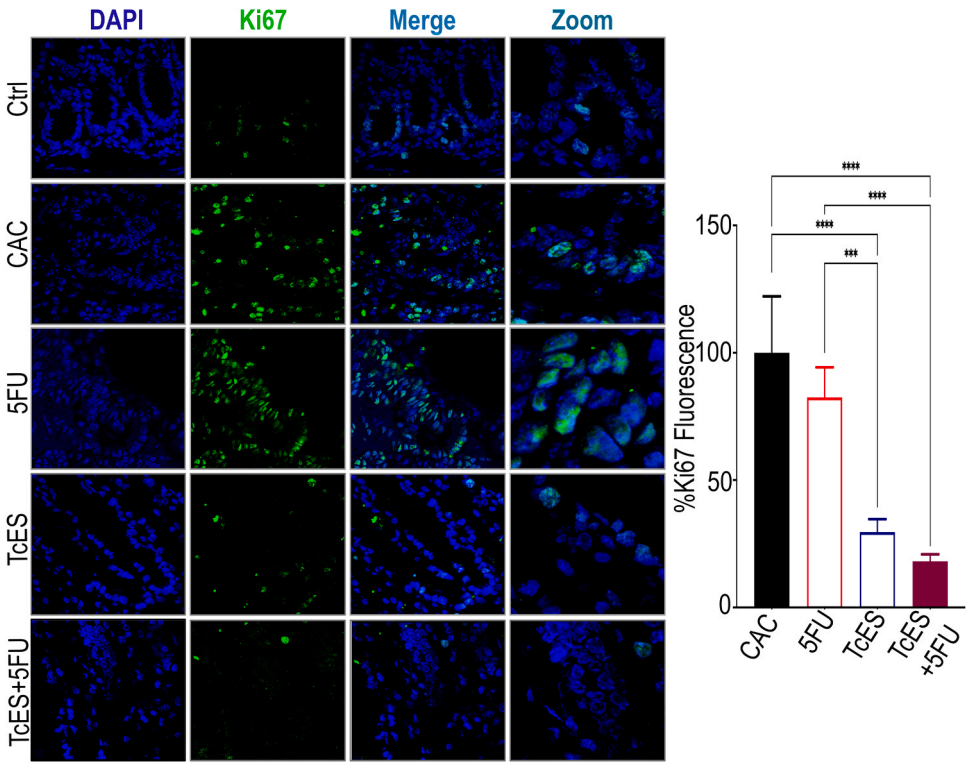

Fig. 4. TcES treatment in the CAC mice regulates the expression of proliferation markers. (A-B) Representative confocal immunofluorescence patterns and quantitative analysis of Ki67, a proliferation marker, in tissues from mice treated and not treated with TcES and 5FU or their combination. Alexa Fluor 488 was utilized as a fluorochrome in the secondary antibody to reveal the Ki67 protein (green), and DAPI was used to visualize nuclei (blue). The arrows in the merged images point to the localization of Ki67 in the nucleus; the bar = 20 μm. The zoom merge of immunofluorescence was obtained at 60X. Data represent three independent experiments with 4–6 mice per group. The data are expressed as the mean ± SE. p value lower than p=0.0001 (****); p=0.001 (***); p = 0.01 (**) and p = 0.05 (*).

3.5. TcES and TcES+5FU treatments regulate the recruitment of CD8 and NK cells to the peritoneum and tumor site

Immune cells, such as $CD8^+$ lymphocytes and natural killer (NK) cells, recognize and eliminate more immunogenic cancer cells in the early stages of tumor development [21,22]. Here, we evaluated the recruitment of $CD8^+$ and NK cells into the peritoneum and colon cancer tissues. Flow cytometric analysis of cells recruited to the peritoneum 90 days after AOM injection showed that mice receiving 5FU and untreated CAC mice displayed a small number of $CD3^+CD8^+$ cells. In contrast, treatment with TcES and TcES+5FU increased the recruitment of $CD3^+CD8^+$ cells by up to threefold compared to that in untreated CAC mice (Figs. 5A and 5B). Similarly, the recruitment of NK cells to the peritoneum by TcES and TcES+5FU treatments was increased by two- and threefold, respectively, compared to that in untreated or 5FU-treated CAC mice (Figs. 5C and 5D). Next, the expression of NK and $CD8^+$ cell markers in the tumoral colon tissues were evaluated to determine whether similar recruitment was also reflected in the colon. We did not find $CD8^+$ cells in the tumor tissues from any of the groups of mice tested (Fig. 5E). Nonetheless, when evaluating the levels of granzyme B1 (GrB1), which is a marker of the cytotoxic activity of T and NK cells, we found that colons of mice treated with TcES monotherapy displayed threefold higher expression of GrB1 than those of CAC-untreated and 5 FU monotherapy-treated mice (Figs. 5E and 5F). Additionally, colon tissues from TcES+5FU mice revealed evident enrichment of GrB1 levels in the infiltrated tissues, which was 13-fold higher than that in the 5FU monotherapy group and 50% higher than that in the TcES group (Figs. 5E and 5F). To define whether NK cells could be responsible for the upregulated GrB1 expression, we evaluated the recruitment of these cells in colon tissues from all groups of mice through immunohistochemistry of CD335, a membrane receptor selectively expressed in NK cells [23]. In the TcES and TcES+5FU groups, NK cells were enriched at the base of the lamina propria, while in the CAC and 5FU groups, these cells were scarcely recruited (Fig. 5G). These data correlated with GrB1 expression in tissues under the same conditions, suggesting that GrB1 expression may be associated with the presence of NK cells.

3.6. The use of TcES as a 5FU adjuvant may favor tumor cell death through apoptosis induction

GrB1 expression is positively associated with tumoral cell death through the induction of apoptosis [24]. To assess whether apoptosis occurred in tumor cells treated with TcES in combination with 5FU, terminal deoxynucleotidyl transferase nick-end-labeling (TUNEL) staining was conducted on paraffin-embedded samples from colonic tumors. Fig. 6A shows that colons of the TcES+5FU group had 80% more TUNEL-positive cells than the CAC group (without treatment) and ~50% more than the 5FU monotherapy group (Fig. 6B). Additionally, the data showed that compared to the CAC group without treatment, the group treated with TcES alone showed no differences in cell death, indicating that the adjuvant effect of TcES enhances the effect of 5FU.

The tumor suppressor protein P53, the product of the TP53 gene, is an essential molecule closely related to the antitumoral activity of 5FU, inducing cell cycle arrest and apoptosis [25]. To determine whether the adjuvant effect of TcES together with 5FU on the induction of tumor cell death was related to the expression and activity of the well-known protein P53, we evaluated its protein levels by Western blotting. P53 expression levels in colon samples from the CAC and 5FU groups were significantly reduced (57%) compared to those in the healthy control group. In contrast, in colon samples from mice receiving TcES+5FU, the expression of P53 was upregulated by ~50% compared to that in mice receiving 5FU and TcES monotherapies (Figs. 6C and 6D), indicating the possible reactivation of P53 by this treatment. This result also correlated

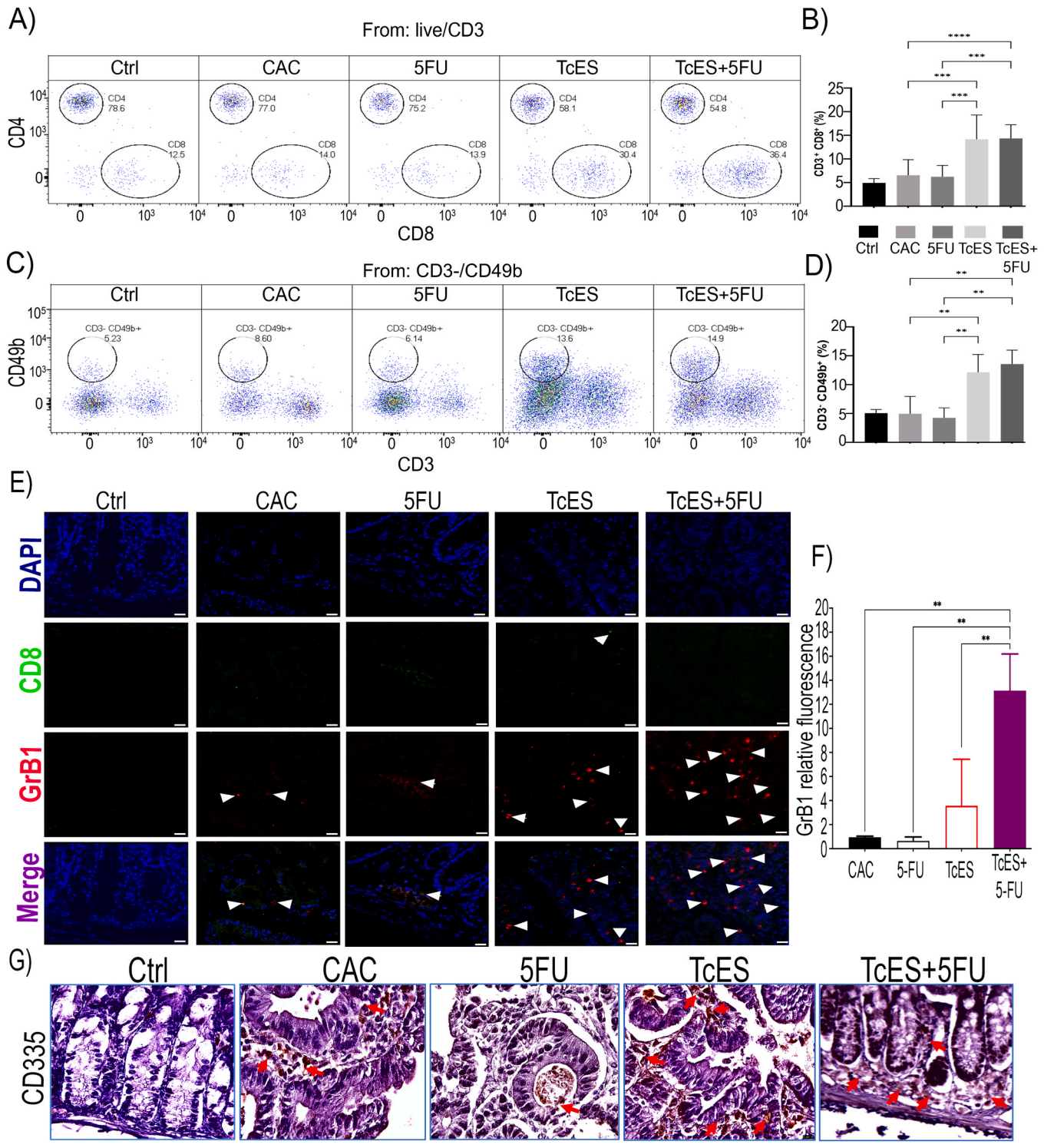

Fig. 5. The combined effect of TcES and 5FU promotes the recruitment and activation of cytolytic immunological cells in the peritoneum and tumor site. (A) Representative dot plots showing T-cell populations in the peritoneum of control and treated mice. (B) Quantitative analysis of the percentage of CD3+/CD8+-gated cells. C) Flow cytometric analysis of CD3-/CD49b+ cells recruited to the peritoneum of mice under different treatment conditions. D) Percentage of $CD3^{-}/CD49b^{+}$-gated cells. (E) Confocal representative images of the immunofluorescence of $CD8^{+}$ (green) and Granzyme B1 (red) counterstained the nucleus with DAPI (blue). The arrows in the merged images point to the localization of $CD8^{+}$ and Granzyme B1 in the tumoral tissues of mice with the treatments utilized in the current work; scale bar = 20 μm. (F) The relative percentage of immunofluorescence of Granzyme B1. (G) NK cell (CD335) infiltration in the colon tissues of Ctrl, CAC, 5FU-, TcES-, and TcES+5FU-treated mice observed by immunohistochemical staining at 40X; the red arrows indicate NK cell localization. Data represent two independent experiments with 4–6 mice per group. The data are expressed as the mean ± SE. p value lower than p=0.0001 (****); p=0.001 (***); p = 0.01 (**) and p = 0.05 (*).

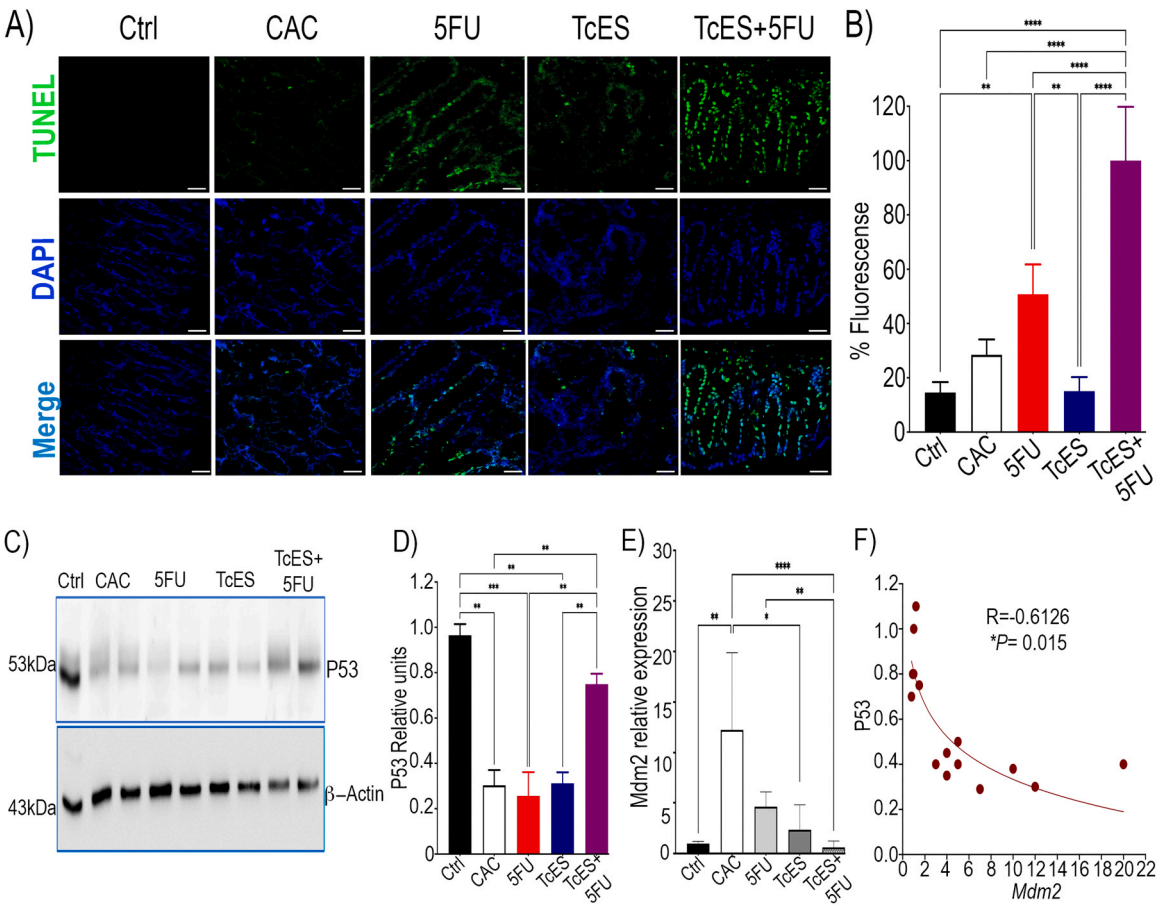

Fig. 6. The adjuvant effect of TcES with 5FU increases apoptosis in colitis-associated colon cancer, inducing the recovery of P53 protein levels. (A) TUNEL assay of colon tissue from CAC mice untreated or treated with 5FU and TcES and in combination (TcES+5FU). Green indicates apoptosis in representative tissue; the nucleus was stained with DAPI (blue). (B) Fluorescence quantification of TUNEL-positive cells in colon tissue. The data are presented as the percentage of mean fluorescence, which was expressed as arbitrary units. Scale bars = 20 μm. (C and D) Representative Western blots and densitometry analysis of total extracts from colon tissues from mice treated with 5FU, TcES, and their combination using an anti-P53 antibody. Densitometric analysis showing data from three Western blots from independent experiments. In all of them, P53 levels were normalized relative to the β-actin protein levels in CAC model mice without treatments. (E) Relative expression levels of *Mdm2* mRNA determined by qPCR in CAC mice without treatment (white), treated with 5FU (faint gray), treated with TcES (dark gray) alone, and treated with TcES+5FU (gridded bar); all relative expression levels were compared with the expression levels in healthy tissues (black bars). (F) Correlation analysis between P53 protein levels and *Mdm2* mRNA expression in tumor tissues treated and non-treated with TcES and 5-FU. The analysis was assessed using Pearson's correlation coefficient. Data represent three independent experiments with 4–6 mice per group. The data are expressed as the mean ± SE. p value lower than p=0.0001 (****); p=0.001 (***); p = 0.01 (**) and p = 0.05 (*).

with the mice group that displayed higher cell death activity (Figs. 6A and 6B). *Mdm2* expression, one of the main negative regulators of P53 [26], was also analyzed in the colons; a significant upregulation of this gene expression was observed in the tissues of untreated CAC mice compared to control healthy mice, while the 5FU mice group revealed a reduction of *Mdm2* levels similar to those of the TcES group. However, TcES+5FU treatment induced a more significant reduction in the expression of *Mdm2,* indicating a combinatory effect of these therapies in down-regulating *Mdm2* (Fig. 6E). Interestingly, the recovery of P53 expression levels correlated with a significant decrease in *Mdm2* mRNA levels (Fig. 6F).

3.7. TcES regulates cell proliferation and migration through P53 signaling in human colon cancer cells

In the previous results of our TcES studies, we determined that TcES reduces the proliferation rate of human colon cancer cells [8]. Thus, we decided to extend the evaluation of P53/P21 axis regulation by TcES treatment and analyzed the protein levels of P21 in human colon cancer cells (HCT-116) and the relationship between TcES and P21. Fig. 7A shows representative Western blots of HCT-116 cells treated with 5FU or TcES as monotherapy, combining TcES+5FU and control cells without treatment (Ctrl). HCT-116 cells treated with 5FU did not express the P21 protein, while cells exposed to TcES exhibited a slight increase in the P21 protein level compared to that in control cells. However, the densitometric analysis obtained from three independent experiments showed that P21 expression levels after treatment with TcES+5FU were 50% higher than those obtained from cells exposed to TcES monotherapy, indicating the possible role of TcES in cell cycle regulation. Next, we evaluated the regulation of malignant markers associated with Y-box binding protein-1 (YB1). YB1 promotes cell proliferation by binding to the promoters of cell cycle-related genes, such as Cyclin D1 [27]. Figs. 7A and 7B show that in cells exposed to TcES, YB1 expression was significantly downregulated, similar to the combination treatment of TcES+5FU, unlike that observed in HCT-116 cells treated with 5FU as monotherapy. These results show that human tumor cells exposed to TcES exhibit downregulated YB1 expression. Given that YB1 participates in cell migration, invasion, and tumorigenicity, we evaluated whether TcES exposure alters the associated changes of YB1 in aggressive features such as cell migration. Three independent wound closure assays were performed with HCT-116 and RKO cells exposed to TcES for 0, 24, 48, and 72 h. The migration of HCT-116 and RKO cells exposed to

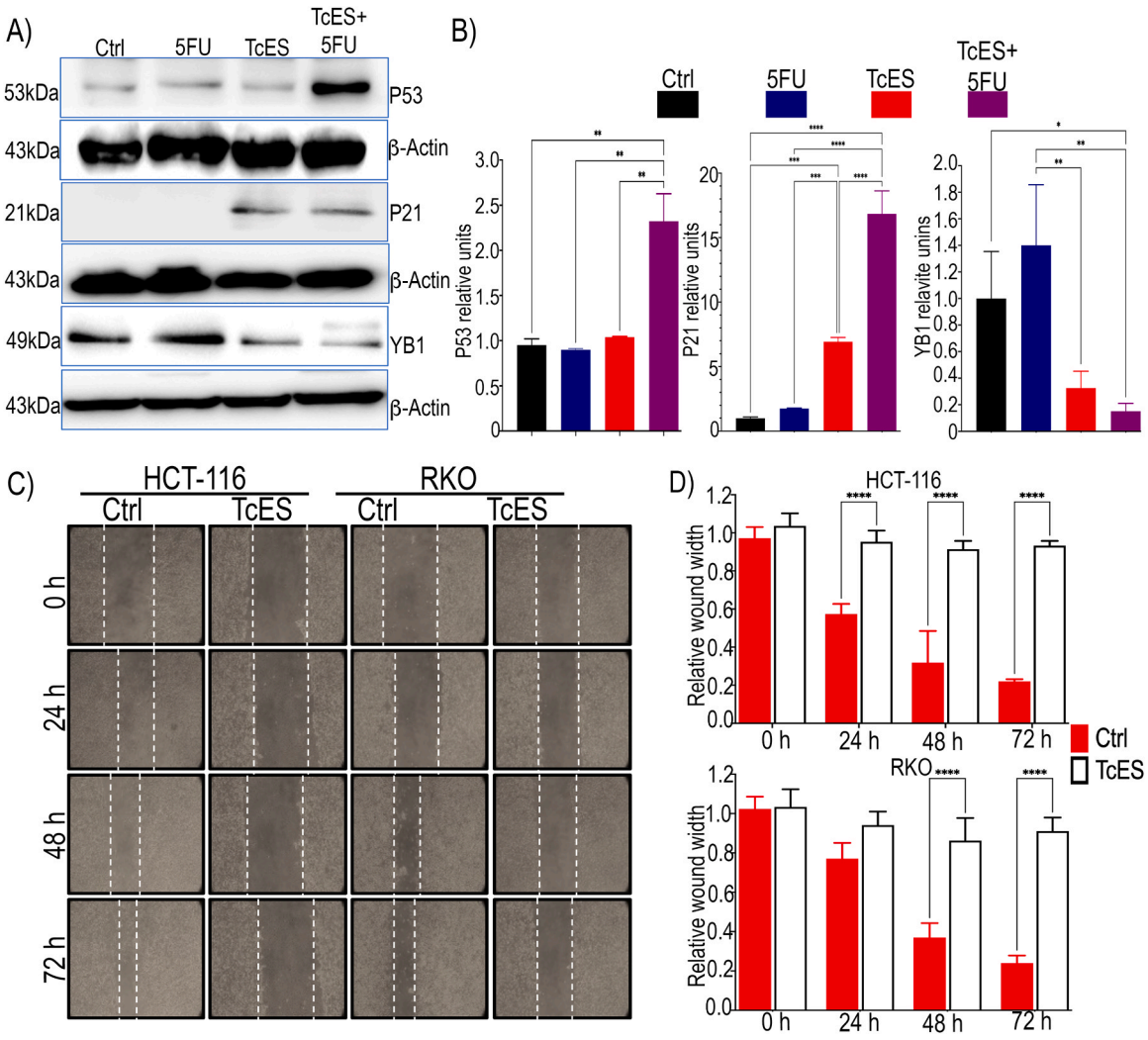

Fig. 7. *Taenia crassiceps* excreted/secreted molecules in human colon cancer cell lines regulate cell proliferation and migration. (A and B) Representative western blots of P53, P21, and YB1 in human HCT-116 tumor cells exposed to 5FU, TcES alone, or the combination of TcES and 5FU. (C and D) Representative migration assay images of HCT-116 and RKO cells treated with TcES at different times (0, 24, 48, and 72 h). The data are expressed as the mean ± SE. p value lower than p=0.0001 (****); p=0.001 (***); p = 0.01 (**) and p = 0.05 (*).

TcES was significantly slower than untreated control cells. Fig. 7C-7D shows that in HCT-116 cells, the effect of TcES on delayed migration was evident after 24 h, in which the control cells covered ~35% of the wound area. In contrast, no changes were observed in RKO cells under the same conditions (24 h). However, at 48 h, the wound in untreated cells was ~60% closed in both cell lines, which increased to 80% at 72 h. Interestingly, neither cell line exposed to TcES significantly reduced wound closure (Figs. 7C and 7D).

4. Discussion

Colorectal cancer remains one of the world's most diagnosed and deadliest types of cancer. It is well known that prevention and prophylactic therapies are the best way to counteract these growing incidence rates of CRC over time. However, we must address the current problem of low patient response rates to therapies in established colon cancer, which contributes to the death of CRC patients. Researchers agree that using 5FU as a unique therapy is insufficient to control CRC [28,29]. Therefore, its combination with other toxic drugs can improve the response rate of up to 50% compared to that of 5FU as a single therapy and is frequently used [5,30]. Nonetheless, the side effects of drug combinations and chemoresistance lead to recurrence and mortality [31]. Hence, it is necessary to explore new alternatives that improve the current therapies, increase the patient response, and prevent the development of chemoresistance and recurrence in CRC patients. The biological IMMODIN (a lyophilized extract from human leucocytes) has recently been used as an adjuvant for 5FU. However, it did not improve the efficacy of 5FU therapy in a mouse CRC model [32].

The use of parasites and their derivatives as alternative therapies remains controversial, but experimental studies have demonstrated a beneficial effect of helminth infections in regulating different inflammatory diseases and types of cancer, including CRC [33,34]. Nevertheless, other works indicate the contrary [35]. A retrospective analysis showed that in patients infected with the helminth *Echinococcus granulosus*, the prevalence of certain types of cancer, such as liver cancer and gastric carcinomas, was lower than that in noninfected patients [36]. In line with these studies, previous work from our group, utilizing a model of colitis-associated colon cancer, established the potential prophylactic effect of the infection and the use of molecules excreted/secreted by *Taenia crassiceps* (TcES), inhibiting inflammatory cytokines related to colon cancer development (IL-1β, TNF-α, IL-33, and IL-17) and suppressing Signal transducer and activator of transcription 3 (STAT3) and Nuclear factor-kappa B (NF-κB) signaling, thus preventing colon tumorigenesis [8] Since the high death rates in CRC are related to an inadequate response to drugs and acquired chemoresistance, we tested TcES as an adjuvant therapy with 5FU in already well-established colon

cancer in the CAC mouse model. We showed here that TcES administered as an adjuvant for 5FU induces a remarkable and significant reduction in the number and size of colon tumors by regulating different mechanisms. This combined therapy downregulated the expression of cytokines involved in the development of colon tumors and resistance to multiple chemotherapeutics, such as *Tgf-β*, *Il-10*, *Tnf-α,* and *Il-17a* [37–39]. Interestingly, both TcES and 5FU as monotherapies or together downregulated the expression of *Il-10* and *Tgf-β* at similar levels, indicating that TcES and 5FU did not exert synergistic effects in this situation. It has been reported that in early growth stages of tumorigenesis, the expression of TGF-β is a potent cell growth inhibitor. However, in oncogenic-activated cells in advanced tumors, the high expression of TGF-β is closely related to tumor progression and malignancy as similarly observed in IL-10 expression in lung cancer patients [40,41]. Thus, is possible to suggest that the effects of TcES in established cancer, negatively regulates molecules involved in the tumor microenvironment, associated with malignancy and progression such asand *Il-10*. As an indirect consequence of inhibition of STAT3 activity, as we previously reported [8]. STAT3 is an active inductor of *Il-10* and is reported necessary for TGF-β-induced cell migration and invasion [42,43]. These findings suggest that treatments that regulate the levels of these cytokines may have a better effect on cancer as show our results. but more detailed studies are necessary to establish the specific mechanism.

TGF-β expression is required for Treg cell development and lineage-specific differentiation, acting as a growth factor that promotes the differentiation of $CD4^+$ T cells into Th17 cells in the presence of IL-6 or IL-21 [44]. Th17 cells release interleukin-17 (IL-17), which enhances the inflammatory environment and correlates with poor patient survival in different cancers, such as hepatocellular carcinoma, biliary tract carcinoma, and colon cancer, among others, promoting resistance to anti-angiogenic therapies [39,45]. We showed here that TcES administration alone and in combination with 5FU critically downregulated *Tnf-α* and *Il-17a* expression. The chronic presence of these inflammatory cytokines in the tumor microenvironment (TME) is closely related to the acquisition of malignancy in tumoral cells and mesenchymal to epithelial transition (EMT) [46]. In this process, the loss of E-cadherin is considered a hallmark at the beginning of EMT, triggering a series of signaling events and major cytoskeletal reorganization [47]. Here, we showed that TcES alone and in combination with 5FU significantly increased the protein levels of E-cadherin in the colon of CAC mice. In the colon of untreated CAC mice or mice receiving only 5FU, the expression level of E-cadherin was significantly reduced, correlating with nuclear translocation of β-catenin, which frequently occurs in both malignancy acquisition and resistance to 5FU treatment, as previously shown in resistant colon cancer cells [48]. Thus, TcES+5FU may modulate both the expression of inflammatory cytokines and the induction of EMT.

β-catenin is the main factor of the canonical Wnt signaling pathway (Wnt/β-catenin). Under tumoral conditions, β-catenin is lost in adherent junctions due to a lack of E-cadherin, accumulating in the cytoplasm and consequently translocating to the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) and activates the downstream target genes of Wnt/β-catenin signaling involved in cell proliferation, migration, and apoptosis inhibition, such as cyclin D1, B-cell leukemia/lymphoma-2 (Bcl-2), and Myelocytomatosis oncogene (c-Myc) [49]. The high expression of Cyclin D1 indicates poor prognosis in colorectal cancer patients, and it may contribute to cisplatin chemoresistance by upregulating NF-κB activity and Bcl2 expression [50]. Here, we found that, compared to the use of 5FU alone, 5FU+TcES may regulate cell proliferation by inhibiting Cyclin D1 expression, in agreement with the significant downregulation in the expression of the traditional proliferation marker Ki67. Our data align with those reported by previous studies on colon cancer cell resistance to 5FU, which showed that curcumin as an alternative treatment downregulated Cyclin D1 expression, decreased proliferation, and induced apoptosis [51].

It is well known that diverse factors influence responses to chemotherapies, and the tumor microenvironment plays a crucial role in chemoresistance and malignant progression in various types of cancer, including CRC [52]. The TME refers to a particular biological environment formed by malignant and nonmalignant cells, such as stromal cells, fibroblasts, immune cells, and their secreted components [53]. $CD8^+$ T cells and NK cells are normally recruited in the TME of CRC [54]. However, during the development of tumor malignancy and resistance to chemotherapy, these cells at the tumor site are frequently lost, or their functions may be altered, generating anergy and tolerance. We demonstrated here that mice receiving TcES alone or in combination with 5FU exhibited increased numbers of $CD8^+$ and NK cells in the peritoneum and NK cells in the colon; this finding correlates with the decreased number and size of colon tumors in these mice, which agrees with a previous report on CRC, where the peritumoral NK infiltrate was associated with improved survival outcomes [55]. Similarly, our data agree with those of CT26 tumor-bearing mice, which had no alteration in the number of CD8 and NK cells after repeated cycles of 5FU treatment in a different model of CRC [56]. We hypothesized that local administration of TcES, in particular, activates the recruitment of immune cells around the site of the tumor, unlike 5FU treatment, and could be considered a secondary effect of TcES in the inhibition of *Tgf*-β expression, which is known to suppress NK cell-mediated antitumor immune responses by inhibiting surface receptors that mediate the recognition of stressed and malignant transformed cells, preventing NK function [57].

$CD8^+$ and NK cells need to be not only recruited to the tumor site but also activated to induce apoptosis due to the immediate release of preformed cytotoxic granules that contain perforin and several proteases called granzymes, of which granzyme B1 (GrB1) is the best characterized [58]. Our data indicate that mice with advanced CAC treated with TcES+5FU exhibit increased expression levels of GrB1 and NK recruitment, indicating that at least one of the cytotoxic immunological populations was recruited and activated in the tumor site. These data suggest that the expression of GrB1 could be a result of the recruitment and activation of NK cells in the tumor site. Thus, cotreatment with TcES and 5FU may rescue the cytotoxic ability of NK cells.

The primary mechanism by which NK cells clear tumor cells is associated with apoptosis induction. We found that although the tissues from mice treated with 5FU as a single therapy exhibited increased levels of tumor cell death compared to those in the tissue from untreated CAC mice, the colons of mice treated with the combination of TcES+5FU displayed significantly higher levels of apoptosis. This effect may correlate with the significant upregulation of GrB1 in the colon under the same conditions. Interestingly, we did not find a correlation between the levels of GrB1 and tumor cell death in CAC mice treated with TcES, indicating that although this improves the recruitment and activation of NK cells at the tumor site, it is not sufficient to induce apoptosis as a single therapy and that other mechanisms could be involved and may be triggered by 5FU.

The wild-type P53 protein regulates the apoptotic pathway triggered by GrB1, and it is well-known that different cancer types carry loss-of-function mutations in the *P53* gene. These alterations in P53 could interfere with GrB1-mediated cell death [59]. The changes in P53 levels and consequent functions could explain the contradictory lack of apoptosis induction in tumors treated with TcES alone even though GrB1 was present, in which the protein levels of P53 were lower than those in the group treated with TcES plus 5FU, which showed higher levels of tumor cell death that correlated with increased levels of P53.

One of the main inhibitors of P53 is the murine double minute 2 (MDM2) oncoprotein. MDM2 overexpression functions as an E3 ubiquitin ligase targeting P53 for proteasomal degradation, leading to poor prognosis and loss of response to different anticancer drugs [60]. Studies in preclinical cancer models in sarcoma, acute leukemia, and colon cancer, among others, indicated that one of the better strategies for WTP53 restoration is to inhibit its binding to MDM2 [61]. Our data support that TcES has adjuvant activity when used with 5FU because it promotes cell death and reduces the number of colon tumors in which

Mdm2 mRNA levels were reduced and P53 protein levels were increased. The downregulated expression of *Mdm2* may allow P53 to be available and exert its activity when needed, as observed in preclinical cancer models utilizing analogs of nutlin, which are specific inhibitors of MDM2 [61]. A more detailed study of the mechanisms by which TcES induces the inhibition of *Mdm2* and restores P53 levels and function is necessary.

Based on these data, we propose two mechanisms by which TcES improves the effect of 5FU on colitis-associated colon cancer: (1) TcES sensitizes tumor cells to 5FU by inhibiting tumor cell proliferation and malignancy, as demonstrated in our in vivo and in vitro models by the reduced levels of β-catenin, the consequent effector cyclin D1, as well as the marker of cell proliferation Ki67. Our experiments using human colon cancer cell lines supported this hypothesis, showing that TcES+5FU treatment positively regulates the P53/P21 axis, which is closely related to cell cycle regulation and apoptosis induction. Additionally, the downregulated expression of YB1, an oncoprotein reported as a marker of poor prognosis in cancer, correlated with lower proliferation and migration of HCT-116 and RKO cells exposed to TcES (Fig. S1 and Fig. 7). The depletion of YB1 reduces cell proliferation and motility in colorectal cancer [62]. These possible mechanisms could be an additional way by which therapy with TcES reduces cell proliferation by inhibiting main markers such as Cyclin D1, which is directly regulated by the binding of YB1 to its promoter [27]. (2) The recovery or upregulated expression of P53 could be the mechanism underlying the improvement of 5FU efficacy by TcES, which first promotes the recruitment and activation of immune cells at the tumor site. The subsequent stimulation with 5FU may lead to apoptosis in the colon tumors of mice treated with this combined therapy. For this reason, although the mice receiving TcES monotherapy showed recruitment of NK cells at the tumor site, they did not show a significant increase in apoptosis, which correlated with the low levels of WT P53 expression. Supporting our data, it has been reported that in mammary and melanoma cells, GrB1 induces cell death by recovering WT P53 expression [59,63]. Here, we found that TcES, together with 5FU, downregulated *Mdm2* expression, which negatively regulates P53, promoting its degradation and quenching, and induced the upregulation of WT P53 in colon cancer tissues that displayed an increase in GrB1 levels, consequently increasing apoptosis (Fig. 8).

The impact of helminths and their molecules on cancer is controversial, but exciting [11,33,34], and the previously performed experiments had been done primarily in vitro and using high doses of helminth antigens. Notably, our experimental data indicate the potential adjuvant role of excreted/secreted molecules of Taenia on CAC treatment in vivo and using lower doses.

5. Conclusions

Taken together our findings, demonstrate for the first time, in vivo and in vitro, that products excreted/secreted by a helminth potentiate the effect of 5FU on reducing colon tumorigenesis through regulating cell proliferation and distinctive markers of malignancy, favoring the recovery of apoptosis induction.

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CRediT authorship contribution statement

Carlos Pérez-Plascencia: Resources, Investigation, Formal analysis. **José L Reyes:** Writing – original draft, Formal analysis. **Marisol I González-González:** Methodology. **Jorge L Ledesma-Torres:** Methodology. **Veronica García-Castillo:** Methodology. **Karen V Fernández-Muñoz:** Methodology. **Cuauhtémoc A Sánchez- Barrera:** Methodology. **Daniela Medina-Reyes:** Methodology. **Luis I Terrazas:** Writing – review & editing, Writing – original draft, Supervision,

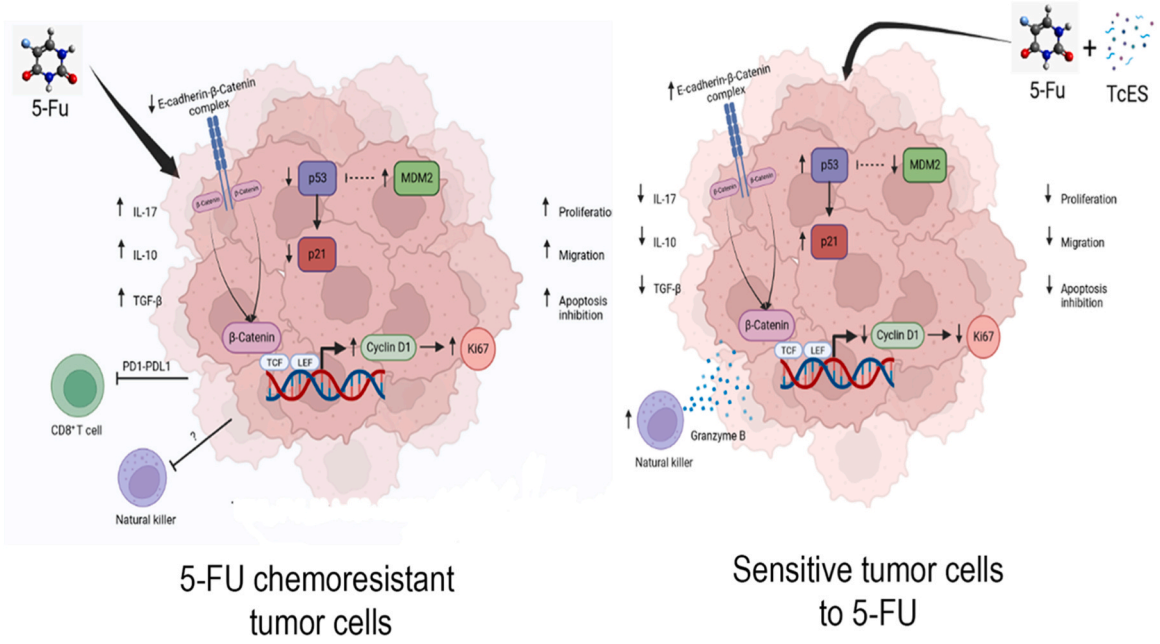

Fig. 8. Graphical model of putative mechanisms triggered by exposure to TcES and subsequent treatment with 5FU in colon cancer. TcES decreases the levels of the pro- and anti-inflammatory cytokines IL-10, TGF-β, TNF-α, and IL-17, which decreases the protein levels of Cyclin D1, β-catenin, and Ki67. Additionally, treatment with TcES+5FU induces the recruitment of NK cells to tumor sites, activating the release of granzyme B1 and, therefore, the apoptosis of tumor cells. This figure was created using BioRender.com.

Resources, Funding acquisition, Formal analysis, Conceptualization. **Monica G Mendoza-Rodriguez:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Marco A Meraz-Rios:** Writing – original draft, Resources. **Felipe Vaca-Paniagua:** Writing – review & editing, Investigation, Formal analysis. **Miriam Rodríguez-Sosa:** Writing – review & editing, Resources, Formal analysis.

Declaration of Competing Interest

All the authors declare no conflict of interest for the current study.

Data Availability

No data was used for the research described in the article.

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Ethics approval and consent to participate

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), under number CE/FESI/042017/1168 (18/04/2017).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116628.

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