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Schistosoma mansoni soluble egg antigen suppresses colorectal cancer growth *in vitro* and *in vivo*

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ABSTRACT

Background: Colorectal cancer (CRC) is the third most common malignant disease around the world. Because the hosts' immunity plays a great part in regulating tumor cells' growth and progression, immunotherapies have therefore aroused great interest in treating cancers. Currently, scientists have investigated the use of *Schistosoma*-derived soluble egg antigens (SEA), which is known as a strong immune modulator, in treating a series of immune-related diseases.
Methods: In this study, we investigated the anti-tumor effect of SEA against CRC using *in vitro* cell lines, HCT-116 and DLD-1, as well as *in vivo* mouse xenograft model. Approaches such as migration assay, invasion assay, and western blotting were done to analyze the anti-tumor effect of SEA. Furthermore, qRT-PCR and ELISA were performed to identify the immune profile of SEA-treated cells as well as SEA-treated xenograft mice.
Results: In vitro studies suggested that SEA can dose-dependently inhibit the growth and progression of HCT-116 and DLD-1 cells. This inhibition was accompanied by a reduction of epithelial-mesenchymal transition (EMT), inflammasome inactivation, and apoptosis. SEA also downregulated the expression of IL-4 and IL-10 in the CRC cells, which may be the reason why their growth and progression were suppressed. *In vivo* studies showed a similar beneficial effect of SEA, as local administration of 25 μg SEA significantly inhibits tumor cell growth. SEA treatment also shifts the host's immunity from a pro-tumorigenic response to an anti-tumor response.
Conclusion: In conclusion, SEA may provide a beneficial effect against CRC, and further investigation may give promise in CRC treatment.

1. Introduction

Colorectal cancer (CRC) is the third most common malignant disease in the world, with its incidence increasing every year.[1] Both genetic and environmental factors are important in CRC development, with genomic and epigenomic instability contributing the most. These include chromosomal instability, microsatellite instability (MSI), non-MSI hypermutability, and global DNA hypomethylation.[2] In addition, the patient's lifestyle[3–5] and their disease status[6–8] also contribute greatly to the pathogenesis of CRC.

The tumor microenvironment (TME) has a decisive role in tumor progression and immune evasion.[9] The TME involves a very complex system, including both the anti-tumor immune responses derived from the host immune cells and the pro-tumor response which is produced by the tumor itself.[9] Once tumor cells were formed in the host, nature killer (NK) cells, T cells, and macrophages were attracted to the site and become reactive to the tumor. These cells can secrete several anti-tumor cytokines such as IFN-γ, IL-2, IL-4, IL-10, and IL-17 [9, 10]. However, the tumor cells can also release certain immunosuppressive substances such as exosomes or cytokines including IL-10 and transforming growth factor β (TGF-β), attracting anti-tumor cells such as Treg and Th17 cells, leading to immune evasion.[10] The cancer cells can as well overexpress or release certain cytokines to promote their progression. As the hosts' immunity plays a great part in regulating tumor cells' growth and progression, immunotherapies have therefore aroused great interest in treating cancers. These include tumor-peptide-based vaccines,[11] agonistic antibodies,[12,13] cytokine-based drugs,[14] and adoptive cell therapy[15] which can re-activate the immune response.

Schistosomiasis is one of the most devastating parasitic diseases caused by *Schistosoma mansoni* (*S. mansoni*), *S. haematobium*, or

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S. japonicum. Once the parasite resides in the human body, they continuously release eggs; while the majority of eggs pass through the feces, a portion of the eggs enter the human circulation and get lodged in various tissues, causing tissue damage. Schistosome eggs, once reside in the host tissues, will consistently secrete soluble egg antigens (SEA).[16,17] The secreted SEA induces a change in the host immune response: from the T helper type 1 (Th1) response to the Th2 response.[18] This predominant Th2 response then evokes a granulomatous inflammation and subsequentially leads to fibrosis.[18] Yet, because of the powerful immunoregulatory action of the SEA, many researchers have tried to apply it to therapeutics to treat various autoimmune and inflammatory diseases such as Graves' disease,[19] asthma,[20,21] diabetes,[22] colitis,[23,24] and even cancers.[25] In addition to SEA, a recent study has also demonstrated the use of cercarial antigens in treating colon cancers.[26] Although the exact mechanism has yet to be clarified, the use of parasite-derived products may introduce a new era of anti-cancer treatment. Therefore, our study here will investigate the potential of using *S. mansoni* SEA against CRC.

2. Materials and methods

2.1. Cell lines and cell culture

Human colorectal cancer cell lines, HCT-116 (ATCC#: CCL-247) and DLD-1 (ATCC#: CCL-221) cells, were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in McCoy's 5a media (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) or Roswell Park Memorial Institute (RPMI) 1640 media (Gibco; Thermo Fisher Scientific) supplemented with 100 U/mL penicillin (Biowest, MO, USA), 100 mg/mL streptomycin (Biowest), and 10 % fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific). All cell lines were handled under standard operating procedures and incubated in a humidified atmosphere of 5 % CO_2 at 37 °C.

2.2. Ethics statement, animals, and parasites

Animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) of Tzu Chi University (No. 110027 and No. 110075) and were conducted under accepted practice standards of the National Institutes of Health (NIH) "Guide for the Care and Use of Laboratory Animals" (DHHS publication No. NIH 85-23, revised 1996).

Eight-week-old, male BALB/c mice were obtained from the National Laboratory Animal Center, Taipei, and were housed in an animal facility at Tzu Chi University in a standard polycarbonate cage on bedding. Food and water were available *ad libitum* and cages were changed weekly. Animals were maintained under a 23°C ± 1°C and a 12-h light/dark cycle condition with 40–60 % humidity.

Puerto Rico strain of *Schistosoma mansoni* (*S. mansoni*) was acquired from the Biomedical Research Institute, Rockville, MD, USA and was maintained in the laboratory as described previously.[27]

2.3. Preparation of Schistosoma mansoni soluble egg antigens (SEA)

Mice infected with *S. mansoni* were terminated at eight weeks post-infection. Livers were collected and blended with sterile saline. Schistosome eggs were separated by passing the blended liver lysate through a sequence of sieves with decreasing pore sizes: 420, 177, 105, and 25 μm. The eggs maintained on the 25 μm sieve were collected in sterile saline and centrifugated at 370×*g* for 2 min. The centrifuged eggs were then resuspended in saline. Soluble egg antigen (SEA) was prepared by homogenizing the eggs with a glass homogenizer and stored at −80 °C until use.

2.4. Cell proliferation assay

For proliferation assay, cells were seeded at a density of 1×10^3 cells/well in 96-well plates. Two hours later the cells were treated with indicated concentration of SEA for 24 or 48 h. The proliferation of cells was determined using WST-1 assay (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. The absorbance of the reaction was measured at a wavelength of 450 nm (nm).

2.5. Colony formation assay

One thousand cells (per well) were seeded in 6-well plates. The cells were grown for 10 days with SEA-containing medium changes every two days. Finally, the cells were washed with PBS, fixed with 75 % ethanol, and stained with 1 % crystal violet. The number of colonies was counted under a microscope.

2.6. Wound healing assay

Cells were seeded at a density of 3×10^5 cells/well in 12-well plates for 24 h. Prior to creating a scratch with a pipette tip, cells were serum-starved overnight. After cell debris was removed by washing with glucose-potassium-sodium phosphate solution (GKNP), cells were maintained in a medium containing 0.5 % FBS and indicated concentrations of SEA. Cell culture was photographed at 0 and 24 h using an Olympus CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan) at 4 × magnification. The area of the remaining wound relative to the initial wound area was determined.

2.7. Transwell invasion assay

Cell invasion was assessed using 24-well cell culture inserts (Corning, USA) with a polyethylene terephthalate membrane with an 8 μm pore size. The upper chamber was coated with Matrigel (100 μg/cm^2; Corning) and incubated at 37 °C overnight for gelling. Briefly, 5×10^5 cells in SEA-containing, FBS-free medium were seeded in the upper chamber, and 750 μL of media supplemented with 10 % FBS were added to the lower chamber. The cells were incubated at 37 °C for 48 h. After that, the chambers were washed twice with PBS, fixed with 3.7 % formaldehyde, permeabilized with 100 % methanol, and stained with 1 % crystal violet. The cells that adhered to the bottom surface of the membrane were photographed, after which cells in five randomly selected fields were counted under a microscope at 4 × objective magnification.

2.8. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

Cells were seeded at a density of 8×10^5 cells/well in a 6-cm-diameter dish. After which the cells were treated with indicated concentrations of SEA for 24 h. Total RNA of the cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNA (5 μg) was used for reverse transcription with RevertAid First Strand cDNA Synthesis Kit (Fermentas International Inc. Ontario, Canada). The qRT-PCR reaction was performed by LabStar SYBR qPCR Kit (Bioline, London, UK) using Roche LightCycler 480 system. Amplification and detection were performed as follows: 45 cycles of denaturation at 95 °C for 15 s, 60 °C for 20 s, and extension at 72 °C for 15 s. The oligonucleotide primers used were shown in Supplementary Table 1. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and gene expression levels were normalized to *GAPDH* control.

2.9. Protein extraction and western blotting

Cells were seeded at a density of 8×10^5 cells/well in a 6-cm-diameter dish. Cells were then treated with indicated concentrations of SEA for 48 h. After washing with PBS, proteins were extracted by RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.). Extracted proteins were separated on 8 % or 10 % SDS-PAGE gels and were transferred to PVDF membranes. Membranes were blocked with 5 % non-fat milk and then

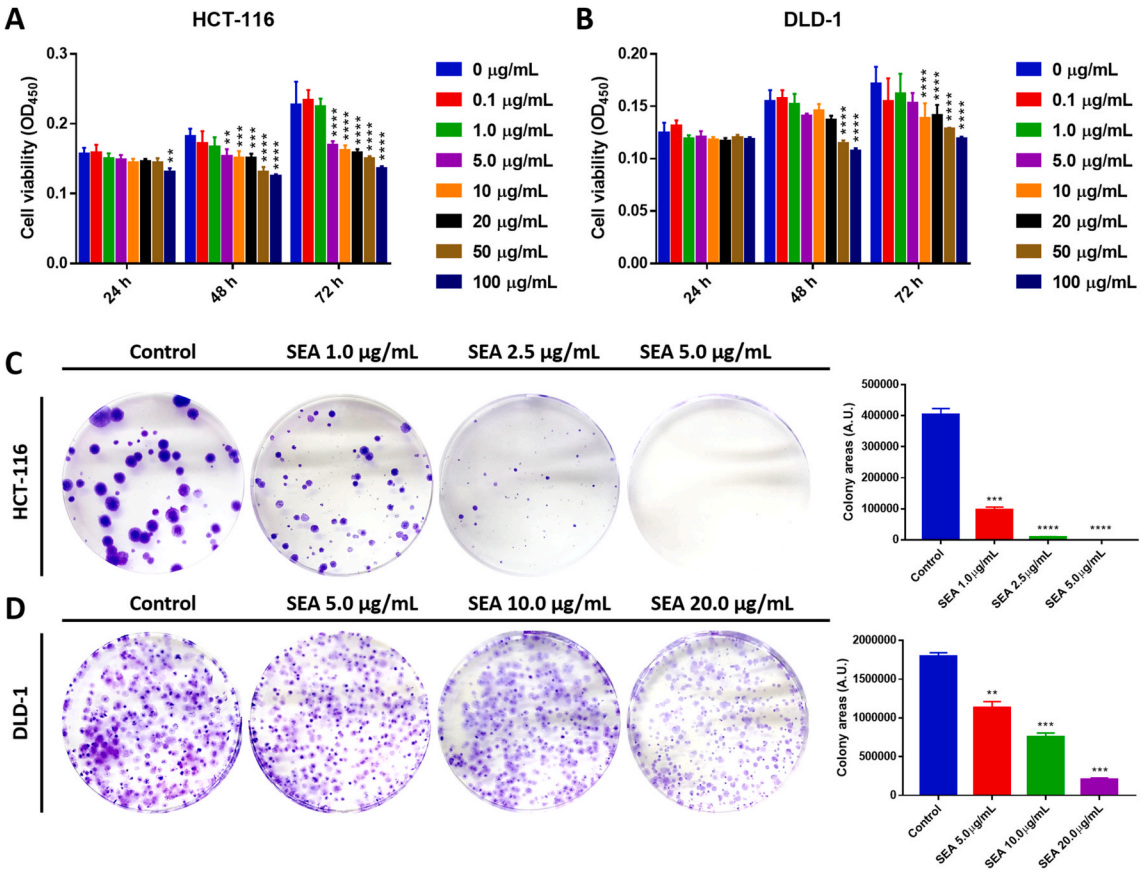

Fig. 1. *S. mansoni* soluble egg antigen (SEA) inhibits the growth of HCT-116 and DLD-1 cells. (A–B) Viability of (A) HCT-116 and (B) DLD-1 cells treated with different concentrations of SEA for 24, 48, and 72 h. SEA dose-dependently inhibits the viability of these cells. (C–D) Representative images of colony formation assay of (C) HCT-116 and (D) DLD-1 cells treated with the indicated concentrations of SEA. Quantification of colony areas was done by Image J software. SEA dose-dependently inhibits the growth of the cells. Data are representative of three independent experiments and values are presented as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ compared to control group. Significance according to one-way ANOVA.

incubated with the following antibodies: α-tubulin (Cat#: GTX628802; GeneTex, Irvine, CA, USA), E-cadherin (Cat#: ab1416; Abcam, Waltham, MA, USA), N-cadherin (Cat#: ab18203; Abcam), Vimentin (Cat#: IR45-137; iReal Biotechnology, Hsinchu City, Taiwan), fibronectin (Cat#: ab268020; Abcam), caspase-3 (Cat#: GTX110543; GeneTex), BCL-2 (Cat#: GTX100064; GeneTex), NLRP3 (Cat#: 19771-1-AP; Proteintech, Rosemont, IL, USA), caspase-1 (Cat#: 22915-1-AP; Proteintech), IL-18 (Cat#: 10663-1-AP; Proteintech), IL-1β (Cat#: 16806-1-AP; Proteintech), and GSDMD (Cat#: SC-393656; Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-IgG (Cat#: AP308P; EMD Millipore, Danvers, MA, USA) or HRP-conjugated rabbit anti-IgG (Cat#: AP307P; EMD Millipore) secondary antibodies prior to the development of the membranes by ECL detection reagent (EMD Millipore). Protein expressions were quantified by Image J (National Institutes of Health, Bethesda, MD, USA) and expressed relative to α-tubulin.

2.10. Tumor xenograft model

1×10^7 DLD-1 cells (suspended in 200 μL PBS) were injected subcutaneously into the right flanks of six-week-old, male BALB/c mice. When the volume of the xenograft tumor reached 80–100 mm^3, the mice were randomly assigned into two groups. Mice in the treated group were injected locally (near the xenograft tumor) with 25 μg SEA (suspended in 100 μL PBS) every two days for 14 days. The control group and the non-treated group were injected with the same volume of vehicle (sterile PBS). Bodyweight and tumor volume were recorded every two days. The tumor volume was measured according to the following equation: $V = \pi/6$ (L × W × H), where "L", "W", and "H" represent the length, width, and height of the xenograft, respectively. Mice were sacrificed 14 days after treatment and tumors were harvested and weighed.

2.11. Tissue processing and hematoxylin and eosin (H&E) staining

Collected tissues were immediately fixed with 10 % formalin for 24 h. After fixation, tissues were dehydrated in a series of graduated changes of alcohols. The procedures were followed by immersion in xylene and infiltration with paraffin. Tissues were then embedded with melted paraffin and the tissue blocks were then sectioned using a microtome. Before proceeding with the hematoxylin and eosin (H&E) staining, the slides were deparaffinized at 60 °C and passed through Sub-X xylene substitute (Leica Biosystems, Richmond, IL, USA), 100 %, 95 %, 75 %, 50 % ethanol, and distilled water. The rehydrated sections were then stained as follows: hematoxylin solution (Merck, Darmstadt, Germany), 70 % ethanol with 1 % HCl, eosin solution, 95 % ethanol, 100 % ethanol, and Sub-X xylene substitute.

2.12. Immunohistochemistry (IHC) staining

Paraffin slides were deparaffinized and rehydrated as described above. Antigens were retrieved by soaking the slides in boiling sodium citrate buffer for 20 min. After antigen recovery, the slides were overlaid with 3 % H_2O_2 for 10 min and 10 % FBS (Gibco, Thermo Fisher Scientific) for 1 h. Thenceforwards, the slides were stained overnight at 4 °C with a Ki-67 (Cat#: A2094; ABclonal) primary antibodies at a dilution of 1:100. After washing, the slides were stained with HRP-conjugated

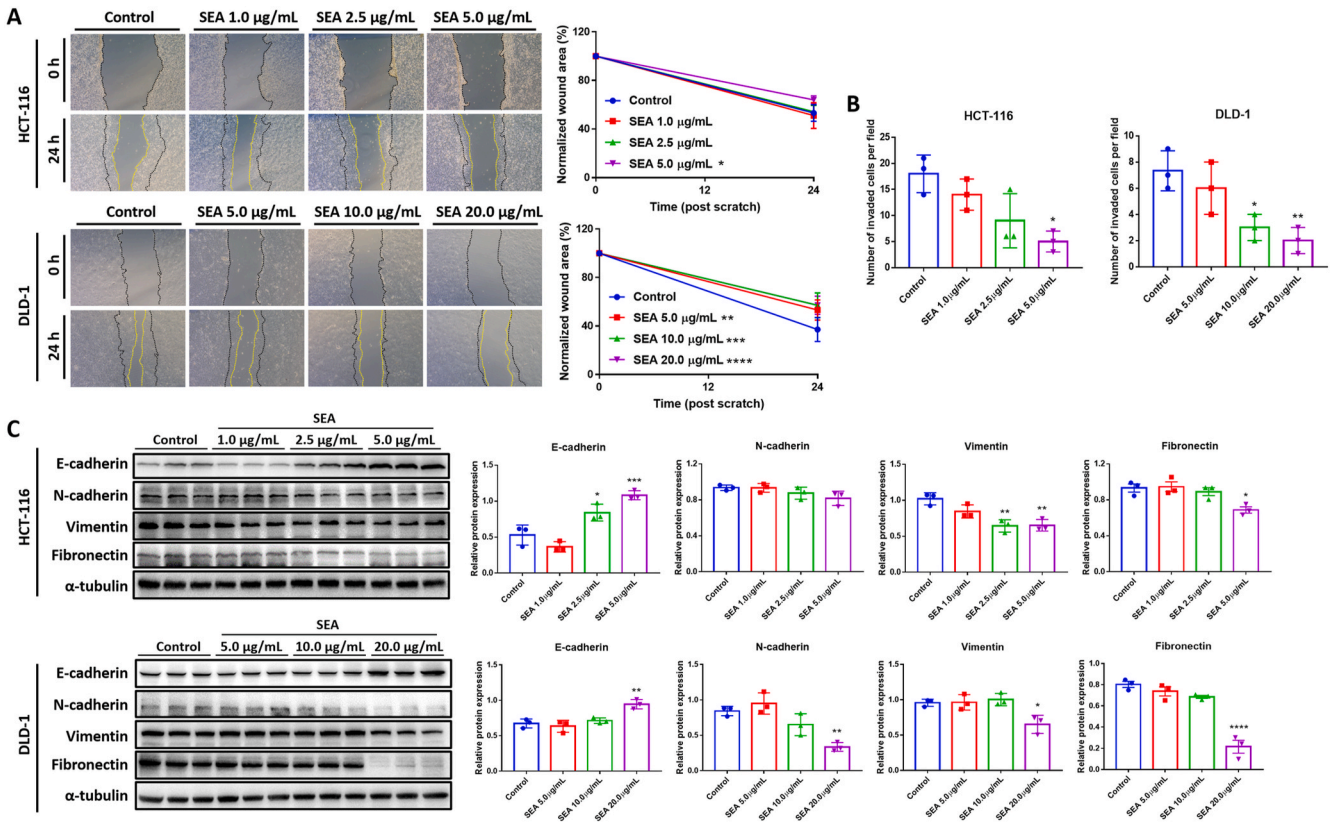

Fig. 2. *S. mansoni* soluble egg antigen (SEA) inhibits the progression of HCT-116 and DLD-1 cells by suppressing epithelial-mesenchymal transition (EMT). (A) Wound healing assay of HCT-116 and DLD-1 cells. Representative images were shown and quantification of the wound areas was done by Image J software. (B) Invasion assay of HCT-116 and DLD-1 cells (see also Supplementary Fig. 1). Quantification of the number of invaded cells per field. (C) Representative Western blot images of EMT markers. Expression levels are relative to that of α-tubulin. Data are representative of three independent experiments and values are presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$ compared to control group. Significance according to one-way ANOVA.

secondary antibody (1:1000 dilution; EMD Millipore) for 30 min and then with freshly prepared 3, 3′-diaminobenzidine (DAB; Thermo Scientific) for 3 min. Sections were then counterstained with Hematoxylin (Merck) and dehydrated with a series of increasing concentrations of ethanol and Sub-X xylene substitute before mounting.

2.13. Enzyme-linked immunoassay (ELISA) for cytokine concentrations

Concentrations of IL-1β, IL-4, IL-10, IL-2, IL-5, and IFN-γ in the culture media or sera were measured using an ELISA kit (Cat#: 432604 for IL-1β; BioLegend, San Diego, CA, USA; Cat#: 88-7711-44 for IL-4, IL-10, IL-2, and IFN-γ; Cat#: 88-7054-22 for IL-5; Thermo Fisher Scientific, Waltham, MA, USA) following the kit's manual. Briefly, 96-well ELISA plates were prepared by coating the plate with 100 μL per well capture antibody overnight at 4 °C. The capture antibody was discarded, wells were washed, and 200 μL per well ELISA/ELISASPOT diluent was added to block the well for 1 h. The wells were then washed and reacted with 100 μL samples or standards for 2 h. After that, plates were washed and 100 μL per well detection antibody was added and incubated for 1 h. The wells were then washed and incubated with 100 μL Avidin-HRP enzyme for 30 min. Posterior to washing, 100 μL per well 3,3′,5,5′- tetramethylbenzidine (TMB) substrate was added for 15 min and at the end, 10 % sulfuric acid was added to each well to terminate the reaction. The optical density of the plate was measured at 450 nm.

2.14. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA). Unless stated otherwise, data are represented as the mean ± standard deviation (S.D.). Mann-Whitney *U* test was used to compare the differences between two groups. One-way analysis of variance (ANOVA) was used to compare multiple groups, followed by Turkey's post-hoc test, for comparisons between groups. A *p*-value <0.05 was considered statistically significant.

3. Results

3.1. Soluble egg antigen inhibits the progression of human colorectal cancer cell lines through inhibition of epithelial-mesenchymal transition

To evaluate the effect of SEA in the tumorigenesis of the two colorectal cancer cells, HCT-116 and DLD-1, WST-1 assay was performed to evaluate the effect of SEA on their cellular proliferation. The results demonstrated that SEA dose-dependently reduced the proliferation of colorectal cancer cells (Fig. 1A–B). Whether SEA affected the growth of colorectal cancer cells was next assessed by detecting their colony formation ability. The results showed that SEA significantly reduced the number of colonies formed (Fig. 1C–D). For the reason that migration and invasion are two of the features that reflect the malignancy of colorectal cancer cells, a wound-healing assay, as well as an invasion assay, were performed. The wound-healing assay showed that only a high concentration (5.0 μg/mL) of SEA significantly inhibits the migration of HCT-116 cells; where all three tested concentrations of SEA significantly inhibit DLD-1 cell migration (Fig. 2A). Similarly, SEA dose-dependently inhibits the invasion of these cancer cells (Fig. 2B; Supplementary Fig. 1). While epithelial-mesenchymal transition (EMT) plays a crucial role in tumor cell progression, we next investigated the expression of certain EMT markers by qRT-PCR and western blotting. The expression of N-cadherin, Vimentin, and fibronectin was markedly

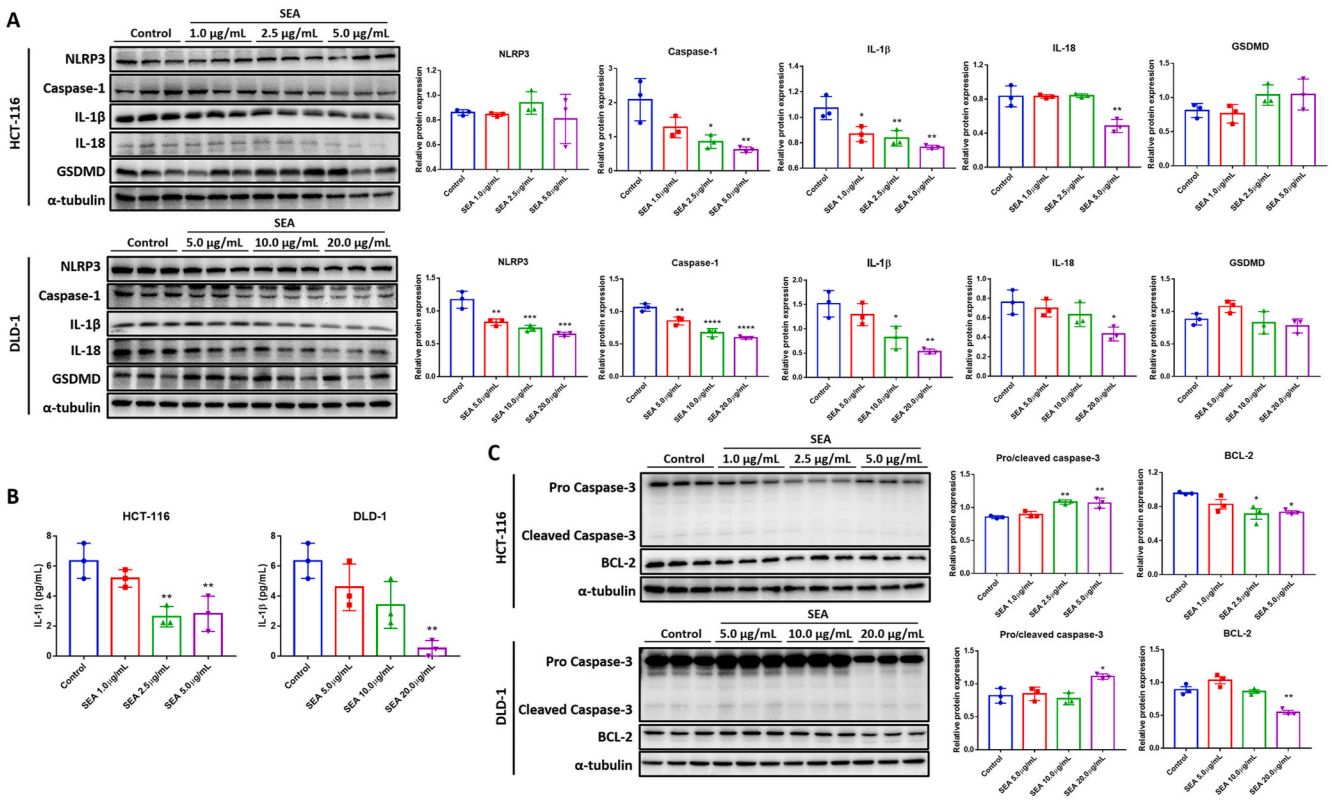

Fig. 3. *S. mansoni* soluble egg antigen (SEA) inhibits inflammasome activation and induces apoptosis in HCT-116 and DLD-1 cells. (A) Representative Western blot images of inflammasome markers. Expression levels are relative to that of α-tubulin. (B) Levels of IL-1β in the medium secreted by HCT-116 and DLD-1 cells. (C) Representative Western blot images of apoptotic markers. Expression levels are relative to that of α-tubulin. Data are representative of three independent experiments and values are presented as mean ± SD. $*p < 0.05$, $**p < 0.01$, and $****p < 0.0001$ compared to control group. Significance according to one-way ANOVA.

decreased by SEA treatment (Fig. 2C; Supplementary Fig. 2A), which was consistent with the results of cell migration and invasion. Accordingly, the expression of E-cadherin was increased by SEA (Fig. 2C; Supplementary Fig. 2A).

3.2. Soluble egg antigen drives NLRP3 inflammasome inactivation and apoptosis in human colorectal cancer cell lines

Emerging evidence reveals that NLRP3 inflammasome and their released inflammatory cytokines play a pro-tumorigenic role in colorectal cancer.[28–31] We therefore hypothesized that SEA also exerts its effect on tumor inflammasome activation. qRT-PCR and western blotting showed that SEA significantly suppresses the expression of NLRP3 inflammasome components in HCT-116 and DLD-1 cells (Fig. 3A; Supplementary Fig. 2B). This reduction of inflammasome expression was accompanied by the decrease of tumor cells-secreted IL-1β (Fig. 3B), suggesting that SEA can inhibit the activation of NLRP3 in colorectal cancers. Inflammasome activation, in addition to activating inflammatory cytokines such as IL-1β and IL-18, also activates a pyroptotic protein, gasdermin D (GSDMD).[32] However, GSDMD expression did not change upon SEA treatment, suggesting pyroptosis did not occur. However, expression of the apoptotic protein caspase-3 increased upon SEA treatment; and this was accompanied by a decreased expression of an anti-apoptotic protein, BCL-2 (Fig. 3C; Supplementary Fig. 2B). These results suggested that SEA resulted in NLRP3 inactivation and apoptosis in CRC.

3.3. Soluble egg antigen regulates cytokine response in human colorectal cancer cell lines

In addition to inflammatory cytokines, other cytokines expressed or secreted by the tumor cells may also affect tumor progression.[14] To establish the immunological mechanism of how SEA affects cancer progression, the expression and secretion of certain cytokines including IFN-γ, IL-2, IL-4, and IL-10 were investigated in SEA-treated colorectal cancer cells. Comparable results can be observed in both HCT-116 and DLD-1 cells: while cancer cell-secreted IFN-γ and IL-2 levels were not changed by SEA treatment (Fig. 4A, B, I, and J), levels of cell-secreted IL-4 and IL-10 was decreased by SEA (Fig. 4C, D, K, and L). Similarly, gene expression of these cytokines in the cancer cells was consistent with their secreted concentration (Fig. 4E–H and M – P).

3.4. Soluble egg antigen inhibits the growth of DLD-1 cells xenograft in mice

To evaluate the actual effect of SEA *in vivo*, a murine subcutaneous xenograft model using subcutaneously-injected DLD-1 cells was treated with SEA every two days. Although the body weight of the xenografted mice was not improved by SEA injection, tumor growth was significantly suppressed (Fig. 5A–C). Tumor weight was also notably reduced in SEA-treated mice (Fig. 5D). Histological analysis showed that tumors harvested from SEA-treated mice revealed a distorted architecture that was infiltrated with numerous immune cells (Fig. 5E). Ki-67 staining also suggested that the proliferation of tumor cells was reduced in mice treated with SEA (Fig. 5F–G). These results may suggest that SEA is beneficial in inhibiting CRC growth, possibly by inducing higher immune cell infiltration which attacks tumor cells.

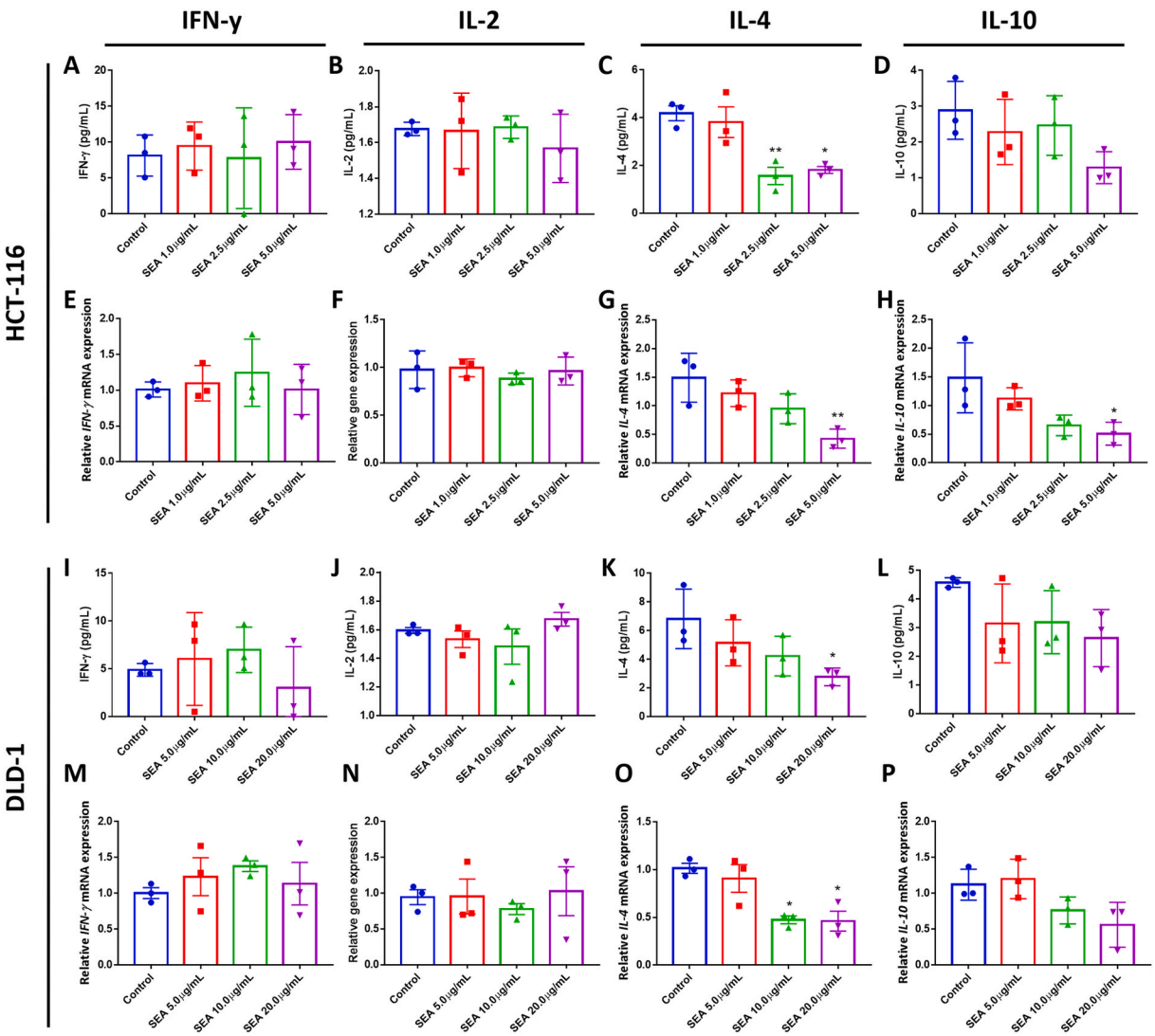

Fig. 4. *S. mansoni* soluble egg antigen (SEA) downregulates intercellular cytokine expression and inhibits the secretion of cytokines. (A-D and I-L) Cytokine levels of (A, I) IFN-γ, (B, J) IL-2, (C, K) IL-4, and (D, L) IL-10 in (A–D) HCT-116 and (I–L) DLD-1 cells, measured in the medium. (E-H and M − P) Relative intracellular mRNA expression of (E, M) IFN-γ, (F, N) IL-2, (G, O) IL-4, and (H, P) IL-10 in (E–H) HCT-116 and (M–P) DLD-1 cells, measured by qRT-PCR. SEA downregulates IL-4 and IL-10 levels in the cells. Data are representative of three independent experiments and values are presented as mean ± SD. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control group. Significance according to one-way ANOVA.

3.5. Soluble egg antigen inhibits epithelial-mesenchymal transition and inflammasome activation, as well as induces apoptosis, of DLD-1 cells xenograft in mice

In accordance with the *in vitro* studies, SEA significantly inhibits EMT of the DLD-1 xenograft, as shown by the increased expression of E-cadherin and decreased N-cadherin and fibronectin (Fig. 6A–C, E). However, vimentin was not changed in expression compared to vehicle control (Fig. 6D). Inflammasome components such as NLRP3, caspase-1, and IL-1β were also inhibited by the SEA treatment (Fig. 6A–F-H). While DLD-1 xenograft significantly increased the mice serum IL-1β, the inhibition of inflammasome was also accompanied by the decreased serum IL-1β concentrations (Fig. 6M). Caspase-3 expression was as well increased in the xenograft, suggesting that the tumor cells undergo apoptosis when treated with SEA (Fig. 6A and K).

3.6. Soluble egg antigen modulates mouse immunity against colorectal cancers

Lastly, we investigated the host immune response as it plays a crucial role in the fight against cancers. DLD-1 xenograft increased serum IL-4 and IL-10 levels in the mice but did not alter levels of IFN-γ and IL-2. By treating SEA to xenografted mice, the mice showed a significantly higher level of IFN-γ (Fig. 7A) and a slight but not significant decrease in IL-2, IL-4, and IL-10 levels (Fig. 7B–D). Of note, SEA treatment to control mice also resulted in a slight but not significant increase in cytokine response, further confirming the immuno-regulatory role of SEA.

4. Discussion

SEA has been shown to modulate the immune response in patients with schistosomiasis, leading to granuloma formation and fibrosis.[33] Yet owing to the powerful immuno-regulatory effect of SEA, many studies have applied SEA in treating autoimmune diseases.[20,23] Since the progression of cancers can be due to their immunoediting process,[34] our study here therefore investigated the therapeutic effect of SEA on CRC.

Our study revealed that SEA treatment significantly inhibits CRC growth and progression both *in vivo* and *in vitro*. This inhibition was accompanied by the reduction of EMT and inflammasome activation; and an increase in tumor cell apoptosis (Figs. 1–3 and 5-6). NLRP3

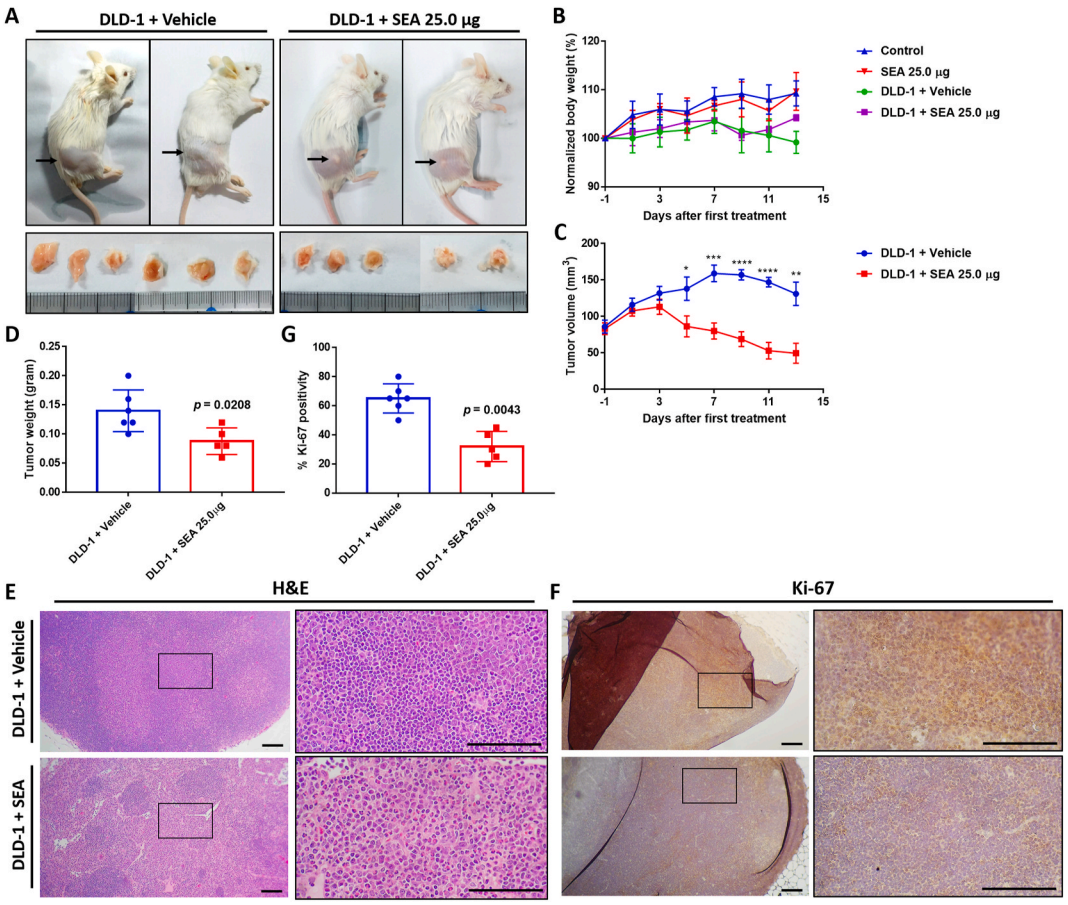

Fig. 5. *S. mansoni* soluble egg antigen (SEA) inhibited tumor growth in DLD-1 xenografted mice. (A) Representative photograph of xenografted mice treated with vehicle (sterile saline) or 25 μg SEA. Treatment was injected near the xenografted tumor every other day for 14 days. (B) Normalized bodyweight of the mice. (C) Tumor volume was measured every other day after the beginning of treatment. (D) Tumor weight. (E) Representative histological image of H&E-stained sections. SEA treatment induces cellular infiltration into the tumors. (F) Representative image of Ki-67-stained sections. (G) Percentage of positive Ki-67 staining. n = 4 control mice; n = 5 SEA-treated mice; n = 6 DLD-1 xenografted mice; and n = 5 SEA-treated DLD-1 xenografted mice. Values are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared between the two groups on the same day. Significance according to Mann–Whitney *U* test.

inflammasomes were considered to be positively associated with the formation and progression of CRC.[31,35–38] The reduction of inflammasome expression was therefore accompanied by reduced tumor growth and progression, as also observed in our results. Interestingly, SEA has never been reported to inhibit inflammasome activation. On the other hand, SEA has been reported to activate inflammasomes in dendritic cells,[39] macrophages,[40] and hepatic stellate cells.[41] While inflammasome activation is a two-step pathway, requiring both priming and activation.[42] In the first step, pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) are recognized by the cells, which activate nuclear factor kappa B (NF-κB) and further production of inflammasome components such as NLRP3 and pro-IL-1β. The second step then involved sensing the PAMPs or DAMPs by the NLRP3, leading to subsequential activation of the inflammasome pathway.[42] While many factors could be involved in the priming step, whether or not the SEA targets the upstream pathway of the inflammasome remains unknown. And this may serve as a base for future studies. In contrast to another study showing SEA-induced pyroptosis,[43] we found that SEA treatment does not affect gasdermin D (GSDMD) expression, both *in vitro* and *in vivo* (Figs. 3 and 6). While activated caspase-1, in addition to IL-1β and IL-18, also cleaves GSDMD into an N-terminal fragment that induces pyroptosis,[42] our results may suggest that even SEA inhibits caspase-1, it does not affect the caspase-1 action on GSDMD.

Further research suggests that SEA can both directly affect the internal mRNA expression of several cytokines in tumor cells (Fig. 4) and indirectly regulate the host immunity against tumor cells (Fig. 7). IFN-γ, IL-2, IL-4, and IL-10 are cytokines that have been shown to modulate CRC growth and progression.[44] Our *in vitro* studies suggest that SEA can downregulate the expression of IL-4 and IL-10, but not IFN-γ and IL-2. Concentration of cell-secreted cytokines also showed similar results (Fig. 4). Several studies have proven that IL-4 is overexpressed during CRC development,[45–47] suggesting that lowering IL-4 expression in the CRC cells may hinder their growth. Higher serum IL-4 levels have also been found in CRC patients with metastasis, suggesting their CRC-promoting role.[48] IL-10 is a pleiotropic cytokine with both pro-tumor and anti-tumor effects.[49] Although some experimental studies suggested that IL-10 is protective against CRC,[50,51] CRC patients with high serum IL-10 were found to be more advanced and have a poorer prognosis.[48,52–54] Mechanistically, while IL-10 is also crucial to activate regulatory T (Treg) cells which limits cancer growth,[55–57] Treg cells may switch from a tumor-protecting phenotype to a tumor-promoting phenotype during CRC development.[58] Regarding the host immunity, a slight but not statistically significant increase in IFN-γ, IL-4, and IL-10 was observed in SEA-injected mice. This result is in line with a previous study, showing that SEA injection alone can slightly increase the production of these cytokines.[59] A similar level of serum IFN-γ and IL-2, and increased IL-4 and IL-10 levels were observed in DLD-1 xenografted mice. Upon SEA treatment, IFN-γ levels significantly increased; while IL-4 and IL-10 levels decreased compared to xenografted mice (Fig. 7). As mentioned, serum IL-4 and IL-10 levels are related to a more advanced CRC, therefore, their reduction by SEA may explain our results

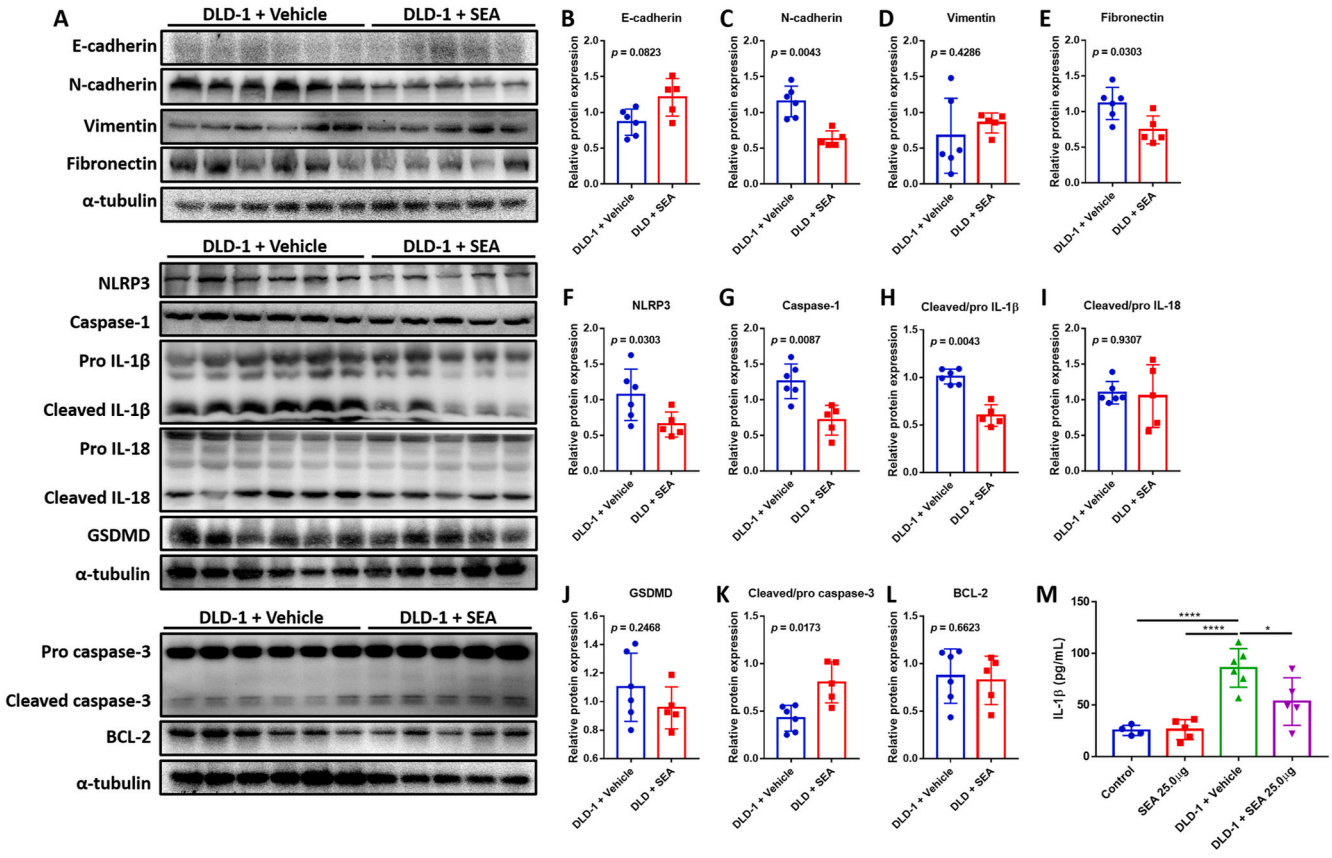

Fig. 6. *S. mansoni* soluble egg antigen (SEA) inhibited epithelial-mesenchymal transition (EMT) and inflammasome activation, and induces apoptosis in DLD-1 xenografted tumors. (A) Representative Western blot images of EMT markers, inflammasome markers, and apoptotic markers. Expression levels of (B–E) EMT markers, (F–J) inflammasome components, and (K–L) apoptotic markers. (M) Serum concentration of IL-1β. n = 4 control mice; n = 5 SEA-treated mice; n = 6 DLD-1 xenografted mice; and n = 5 SEA-treated DLD-1 xenografted mice. Values are presented as mean ± SD. $^{*}p < 0.05$ and $^{****}p < 0.0001$ compared between groups. Significance according to (B–L) Mann–Whitney *U* test or (M) one-way ANOVA.

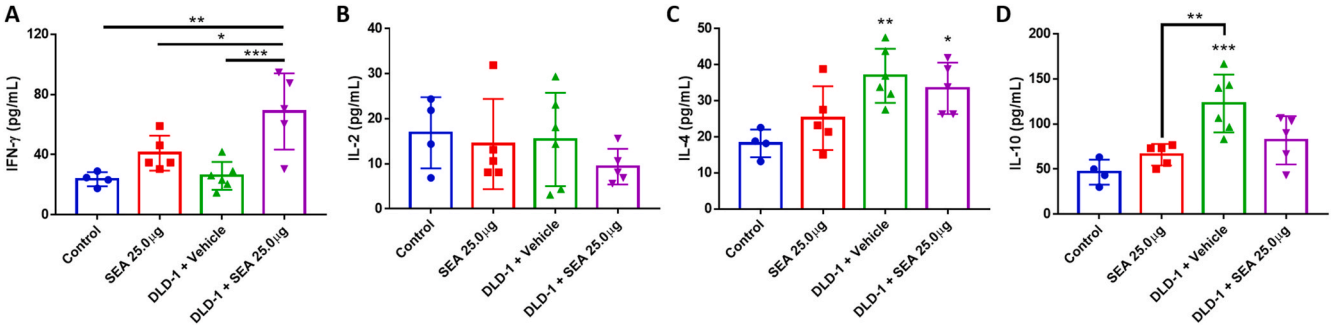

Fig. 7. *S. mansoni* soluble egg antigen (SEA) regulates the immunity of DLD-1 xenografted mice. Serum level of (A) IFN-γ, (B) IL-2, (C) IL-4, and (D) IL-10. n = 4 control mice; n = 5 SEA-treated mice; n = 6 DLD-1 xenografted mice; and n = 5 SEA-treated DLD-1 xenografted mice. Values are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control or between groups. Significance according to one-way ANOVA.

showing the reduction of tumor growth. IFN-γ promotes $CD8^{+}$ T cell-, NK cell-, and macrophage-mediated cytotoxicity against tumor cells[60] and has been shown to induce an anti-tumor response in CRC patients.[48,61] Therefore, the robust increase of serum IFN-γ may stimulate an anti-tumor response, leading to a decrease in tumor growth. On the other hand, IL-2 levels were not changed in both xenografted mice or SEA-treated xenografted mice. The unchanged level in SEA-treated mice may suggest that SEA did not affect the host immunity in an IL-2 axis.

In addition to tissue fibrosis, schistosomiasis has also been shown to be related to human cancers.[62–65] While *S. haematobium* is already proven as a definite carcinogenic agent to humans leading to bladder cancer, there are only limited shreds of evidence that *S. mansoni* or *S. japonicum* is carcinogenic to human CRC. While several reports described the associations of *S. mansoni* with CRC,[62,63,66–68] some studies deny the association between these two diseases.[69–73] Although we cannot ignore the fact that antigens released by the tumor tissue-deposited eggs could lead to a sequence of pathological and molecular events which may lead to a more severe and advanced CRC,[63,66,74,75] we should also consider the differences in the overall effect of the schistosome eggs on CRC and that of purified SEA on CRC. It is also plausible that the SEA may contain numerous substances that could lead to different effects on the cancer cells. This is therefore

another important issue for future research.

CRediT authorship contribution statement

Ho Yin Pekkle Lam: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. **Ting-Ruei Liang:** Data curation, Methodology, Software. **Shinn-Jong Jiang:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. **Shih-Yi Peng:** Conceptualization, Data curation, Funding acquisition, Writing – review & editing.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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