Immune checkpoints such as programmed death-1 (PD-1) have been proven as antitumor targets by enhancing cytotoxic T cell activity. All immune checkpoint blockades are antibody therapeutics that have large size and high affinity, as well as known immune-related side effects and low responses. To overcome the limitation of antibody therapeutics, we have explored PD-1/PD-L1 (programmed death-ligand 1) blockades in traditional oriental medicine, which has a long history but has not yet studied PD-1/PD-L1 blockades. Sanguisorbae Radix extract (SRE) blocked PD-1 and PD-L1 binding in competitive ELISA. SRE effectively inhibited the PD-1/PD-L1 interaction, thereby improving T cell receptor (TCR) signaling and the NFAT-mediated luciferase activity of T cells. SRE treatment reduced tumor growth in the humanized PD-L1 MC38 cell allograft humanized PD-1 mouse model. Additionally, the combination of SRE and pembrolizumab (anti-PD-1 antibody) suppressed tumor growth and increased infiltrated cytotoxic T cells to a greater extent did either agent alone. This study showed that SRE alone has anticancer effects via PD-1/PD-L1 blockade and that the combination therapy of SRE and pembrolizumab has enhanced immuno-oncologic effects.

Keywords: PD-1, PD-L1, Sanguisorbae Radix, cancer immunology, humanized PD-1 mice, tumor-infiltrating CD8+ T cell

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in terms of morbidity and mortality around the world (1). The treatment of advanced CRC has applied chemotherapy, including 5-fluorouracil, oxaliplatin, and irinotecan, and targeted therapy, including bevacizumab and cetuximab, but these agents have resistance, dose-limiting side effects, and are strongly toxic to
normal cells (2, 3). As an improvement over these agents, immunotherapy has been developed as an effective treatment, increasing the strength of the immune system against malignant tumors in patients with CRC along with microsatellite types or mismatch-repair deficiency (4). In immunotherapy, immune checkpoint inhibitors (ICIs) targeting PD-1 or PD-L1 in several human cancers have recently been demonstrated. To date, six ICIs have been approved by the FDA, including pembrolizumab, nivolumab, cemiplimab, atezolizumab, avelumab, and durvalumab (5). The programmed death-1 programmed death-ligand 1 (PD-1/PD-L1) signaling axis can be important to tumor survival and development (6). The PD-L1 transmembrane protein is known to express on the surface of CRC, and its binding with PD-1 leads to the escape of cancer cells from immune-mediated destruction, thereby enhancing cancer cell growth (7). PD-1 is a crucial immune checkpoint molecule and is mainly expressed on the tumor-infiltrating T lymphocytes (TILs), including CD4+ T cells (helper T cells) and CD8+ T cells (cytotoxic T lymphocytes, CTLs) (8). TILs activated by the blocking of the PD-1/PD-L1 interaction release antitumor cytokines containing interleukin-2 (IL-2) and interferon (IFN)-γ, which indirectly help the immune system fight cancer cells (6). In particular, CTLs can directly induce cancer cell death by releasing lytic granules, such as the perforin (PRF) protein, which punches holes in the cancer cell membrane (9). Immunomodulatory activity according to the blocking of the PD-1/PD-L1 interaction enhances the antitumor activity of CTLs in the tumor microenvironment.

Recently, anti-PD-1 medications, such as pembrolizumab and nivolumab, in combination with chemoradiotherapy or targeted therapy has been reported to effectively inhibit the PD-1/PD-L1 interaction in CRC (10). Although these clinical therapies have the advantage of increasing survival rate in patients with CRC, their strong toxicity causes several adverse effects, they have a long half-life, and are expensive to produce (3). Use of novel supplementary therapies, including those from natural sources, is therefore imperative. Compared to clinical drugs, these are rapidly absorbed in the human body, and doses can be easily adjusted, raising the expectation of cure by enhancing patients' long-term survival rate (11). Medicinal herbs containing abundant bioactive ingredients have been utilized as CRC therapeutics to overcome the toxicity and resistance of clinical anticancer drugs (12). Previous investigations regarding the targeting of PD-1/PD-L1 have focused on antibody drugs for CRC immunotherapy but not on known traditional medicinal plants. To discover the more effective CRC immunotherapeutics, we focused on natural products to find combinatorial ICIs with potent synergistic efficacies on the PD-1/PD-L1 interaction.

Sanguisorbae Radix (SR), the dried root of *Sanguisorba officinalis*, also known as great burnet, is a traditional herbal medicine used to treat diarrhea, chronic intestinal inflammation, duodenal ulcers, and internal hemorrhage (13). Recently, multiple studies have reported its diverse pharmacological actions, including antiallergic, anti-inflammatory, antiobesity, and anticancerous (14–17). Although several studies have shown that SR extract (SRE) suppresses both *in vivo* and *in vitro* CRC growth (18, 19), to our knowledge, no literature has reported the antitumor effects of SRE on targeting the PD-1/PD-L1 signaling axis.

Ongoing screening of PD-1/PD-L1 inhibitor from herbal medicine, we found that SRE is a potent inhibitor of PD-1/PD-L1 interaction by *in vitro* competitive ELISA and cell-based luciferase assay. Additionally, we established the antitumor effect of SRE in combination with anti-PD-1 antibodies using a humanized PD-L1 MC38 CRC cell-bearing humanized PD-1 knockin mouse model. That is distinguished studies for CRC immunotherapies targeting human PD-1/PD-L1 in the animal model. Based on this investigation, we proposed a novel combination strategy to improve the effectiveness of immunotherapy by using SRE in cancer patients.

**MATERIALS AND METHODS**

**Preparation of Plant Materials**

SR was supplied by the National Development Institute of Korean Medicine (NIKOM, Gyeongsan, Korea). The dried whole plant (2.0 kg) was extracted with 5 L of 70% ethanol for 1 hour, three times. The extract was percolated with filter paper (3 mm; Whatman PLC, Kent, UK), condensed using a rotary evaporator (Buchi, Swiss), and lyophilized using a freeze dryer (Eyela, Japan). The extract powder (285.41 g; yield 14.27% abbreviated as SRE) was dissolved in 50% dimethyl sulfoxide (DMSO) (Eyela, Japan). The extract powder (285.41 g; yield 14.27% abbreviated as SRE) was dissolved in 50% dimethyl sulfoxide (DMSO) and diluted with culture medium (100 mg/mL) to use in *in vitro* experiments.

**PD-1/PD-L1 Competitive ELISA**

PD-1/PD-L1 competitive ELISA (#72005, BPS Bioscience, San Diego, CA, USA) was performed as per the manufacturer’s protocol. As a positive control, an anti-PD-1 neutralizing antibody (#71120) was purchased from BPS Bioscience. Briefly, recombinant hPD-L1 protein (#71104, BPS Bioscience) was coated on the plates (0.32 cm², #3917, Corning, New York, NY, USA) at 1 μg/mL with phosphate-buffered saline (PBS, pH 7.4) and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 2% (w/v) bovine serum albumin for 1 hour at room temperature (RT). The biotinylated hPD-1 (#71109, BPS Bioscience) of 0.5 μg/mL was added to each well and incubated for 2 hours at RT. The horseradish peroxidase (HRP)-conjugated streptavidin (#554066, BD Biosciences, San Jose, CA, USA) of 0.2 mg/mL was added to each well and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 2% (w/v) bovine serum albumin for 1 hour at room temperature (RT). The biotinylated hPD-1 (#71109, BPS Bioscience) of 0.5 μg/mL was added to each well and incubated for 2 hours at RT. The horseradish peroxidase (HRP)-conjugated streptavidin (#554066, BD Biosciences, San Jose, CA, USA) of 0.2 μg/mL was added to each well and incubated for 1 hour at RT. The relative chemiluminescence was measured using a SpectraMax L microplate reader (Molecular Devices, San Jose, CA, USA).

**Cell Culture**

Recombinant Jurkat T cells expressing human PD-1 and NFAT reporter gene (#60535, hPD-1/NFAT Jurkat T cells) and recombinant CHO-K1 cells expressing human PD-L1 and T cell receptor (TCR) activator (#60536, hPD-L1/TCR CHO-K1 cells) were purchased from BPS Bioscience. The hPD-1/NFAT
Jurkat T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin). The hPD-L1/TCR CHO-K1 cells were maintained in Ham’s F-12 medium supplemented with 10% (v/v) heat-inactivated FBS and antibiotics. These cells were cultured in a complete medium with Geneticin (1 mg/mL) and Hygromycin B (200 µg/mL) to maintain stable cells containing genetic constructs. MC38 cells expressing human PD-L1 (hPD-L1 MC38 cells), derived from C57BL/6 murine colorectal adenocarcinoma, were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China). The hPD-L1 MC38 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% (v/v) heat-inactivated FBS, antibiotics, and Hygromycin B (50 µg/mL). The cells were incubated in a humidified incubator at 37°C under a 5% CO2 atmosphere before the experiments. These solutions for cell culture were purchased from Hyclone Laboratories, Inc. (GE Healthcare Life Sciences, Chicago, IL, USA).

**Isolation and Culture of Murine Splenocytes and Tumor-Infiltrating CD8+ T Cells**

Splenocytes were isolated from the spleens of hPD-L1 MC38 cell-bearing hPD-1 knockin mice. The single-cell suspension of splenocytes was obtained by first filtering through a 100-μm and 40-μm cell strainer (SPL Life Sciences, Pocheon, Korea) and then adding ammonium-chloride-potassium lysing buffer (Lonza, Basel, Switzerland) to remove red blood cells. Tumor-infiltrating CD8+ T cells were isolated from tumor tissues of hPD-L1 MC38 cell-bearing hPD-1 knockin mice. The single-cell suspension of hPD-L1 MC38 tumor tissues was obtained by digesting the tissues with collagenase (0.5 mg/mL collagenase IV) for 1 hour at 37°C and then filtering through 100-μm and 40-μm cell strainer. The hPD-L1 MC38 tumor-infiltrating CD8+ T cells was purified by immunomagnetic negative selection (#19853, STEMCELL Technologies, Inc., Vancouver, Canada). The cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS and antibiotics.

**T Cell-Mediated Killing of Cancer Cells by Coculture System**

Murine lymphocytes and tumor-infiltrating CD8+ T cells (1 × 10^6 cells/9.5 cm^2) as effector cells were activated with Dynabeads T-Activator CD3/CD28 (Life Technologies, Carlsbad, CA, USA) for 72 hours at 37°C. Murine hPD-L1 MC38 cells were stained with CellTrace™ Far Red Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA, USA). The hPD-L1 MC38 cells (5 × 10^4 cells/1.9 cm^2) as target cells were treated with IFN-γ (10 ng/mL) for triggering reactive expression of PD-L1 for 24 hours at 37°C. The hPD-L1 MC38 cells were cocultured with the activated CD8+ T cells (2.5 × 10^5 cells/1.9 cm^2) at an effector cell-to-target cell ratio of 5:1 or with splenocytes (5 × 10^5 cells/1.9 cm^2) at an effector cell-to-target cell ratio of 10:1 and the indicated concentrations (0–50 μg/mL) of SRE for 72 hours at 37°C. After 72 hours, the plates were washed with PBS, the remaining attached live cancer cells were stained with crystal violet solution and measured using a SpectraMax i3 microplate reader at 540 nm. The cocultured hPD-L1 MC38 cells were observed using fluorescence microscopy (Olympus, Tokyo, Japan) and analyzed using a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

**LDH Cytotoxicity Assay**

The lactate dehydrogenase (LDH) liberated from the target cells via effector cells was measured using LDH cytotoxicity assay (#ab65393, Abcam, Cambridge, UK). Briefly, the cell culture medium was mixed with WST Substrate Mix and incubated for 30 min at RT. The reaction was stopped by adding a stop solution, and the absorbance of the formazan products was measured using a SpectraMax i3 microplate reader at 450 nm.

**IL-2 Measurement Assay**

The amount of IL-2 released by activated T cells in the coculture supernatants was measured using a sandwich ELISA.
(#555148, BD Biosciences) according to the manufacturer’s protocol. Briefly, an anti-mouse IL-2 monoclonal antibody was coated on the plates (0.32 cm², #3590, Corning) with 0.1 M sodium carbonate (pH 9.5) and incubated overnight at 4°C. The plates were washed with PBS-T and blocked with PBS containing 10% (w/v) FBS for 1 hour at RT. The biotinylated IL-2 antibody and streptavidin-HRP were added to each well and incubated for 1 hour at RT. The relative absorbance was measured using a SpectraMax i3 microplate reader at 450 nm.

**PRF1 Measurement Assay**

The concentrations of PRF1 released by activated T cells in the cell coculture supernatants were quantified using a sandwich ELISA (NBP3-00452, Novus Biologicals, Centennial, CO, USA) according to the manufacturer’s protocol. Briefly, the standard samples were added to the coating plates and incubated for 90 min at 37°C. The biotinylated PRF1 and HRP-streptavidin were added to each well, then incubated for 1 hour at 37°C. The relative absorbance was measured using a SpectraMax i3 microplate reader at 450 nm.

**Tumor Allograft and SRE Treatment**

Human PD-1 L1 MC38 cells (3 × 10⁵ cells/200 µL PBS) were injected into the dorsal subcutaneous skin of C57BL/6J/Ma mice knockin mice. Tumor growth was monitored and tumor size was measured using digital calipers (Hi-Tech Diamond, Westmont, IL, USA), and tumor volume was calculated according to the formula (length × width²)/2. The tumor volumes reached 20 mm³ (day 10), and mice were randomized into groups of six animals per group. The vehicle (PBS) group and SRE-treated groups were orally administered 100 mg/kg and 300 mg/kg of SRE in 100 µL PBS/20 g once daily using oral zonde for 17 days, respectively. The anti-PD-1 antibody-treated group was administered 2.5 mg/kg of pembrolizumab in 100 µL PBS/20 g via intraperitoneal injection on days 1, 4, 8, and 15. All mice were euthanized for analysis 18 days after treatment.

**Blood Biochemistry**

Blood sera were collected via cardiac puncture of humanized PD-1 mice treated with SRE and anti-PD-1 antibodies using blood-collection tubes (#365967, BD Biosciences). The levels of AST, ALT, blood urea nitrogen (BUN), and creatinine were analyzed using biochemical analyzer XL 200 (Erba Lachema s.r.o, Mannheim, Germany).

**Immunohistochemistry**

For immunohistochemical analysis, the tumor tissues were fixed with 10% formalin in PBS and embedded in paraffin. The paraffin sections were incubated with a primary antibody against the CD8 (#98941, Cell Signaling Technology, Danvers, MA, USA) and PRF (#31647, Cell Signaling Technology). The tissue slides were visualized via DAKO EnVision kit (#K5007, DAKO, Jena, Germany). The sections were counterstained with hematoxylin. Hematoxylin-eosin stain was performed for histopathological examination of tumor tissues. Images were observed using an Olympus BX53 microscope and XC10 microscopic digital camera (Tokyo, Japan).

**Statistical Analysis**

The data are presented as the mean ± standard deviation and were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The difference of mean values was analyzed by one-way ANOVA, followed by Tukey’s post hoc test, which was used for comparisons between multiple groups, as indicated. Differences with a p-value <0.05 were considered significant. All experiments except those in the animal studies were conducted on at least three independent occasions.

**RESULTS**

**SRE Blockade of PD-1/PD-L1 Interaction**

To investigate the effect of SRE on PD-1/PD-L1 blockade, we experimented with competitive PD-1/PD-L1 ELISA-binding assays. The results showed that SRE blocked the binding of PD-1 to PD-L1 in a dose-dependent manner (Figure 1A). In controls, anti-PD-1 antibodies also inhibited PD-1/PD-L1 interaction in a concentration-dependent manner (Figure 1B). The 50% inhibitory concentration (IC₅₀) values of SRE and anti-PD-1 antibodies were 50.10 ± 15.14 µg/mL and 1.49 ± 20.59 µg/mL, respectively.

**SRE Blockade of PD-1/PD-L1 in Luciferase Assay**

To elucidate the effect of SRE on TCR activation, we conducted coculture systems using human PD-1-expressing Jurkat T cells that expressed NFAT-derived luciferase reporter (hPD-1/NFAT Jurkat cells) and human PD-L1-expressing aAPC/CHO-K1 cells designed to activate cognate TCR (hPD-1/TCR CHO-K1 cells). The cell culture model was established to evaluate the effect of PD-1/PD-L1 blockade (20). To examine the cytotoxic effects of SRE, hPD-1 effector cells and hPD-L1/TCR CHO-K1 cells were treated with the indicated concentration (0–50 µg/mL) of SRE for 24 hours. SRE had no cytotoxic effect on cells up to the concentration of 50 µg/mL (Figures 2A, B). The 50% cytotoxic concentration (CC₅₀) values of SRE on the hPD-1/NFAT Jurkat cells and hPD-L1/TCR CHO-K1 cells for 24 hours were 97.03 ± 11.51 µg/mL and 139.45 ± 11.49 µg/mL, respectively. The effect of SRE in coculture cell model systems using a PD-1/PD-L1 blockade bioassay was examined. The 50% effective concentration (EC₅₀) values of SRE and anti-PD-1 antibodies were 4.974 ± 0.04 µg/mL and 0.239 ± 0.08 µg/mL, respectively (Figures 2C, D). These findings suggest that SRE effectively inhibited the PD-1/PD-L1 interaction, thereby improving TCR signaling and NFAT-mediated luciferase activity of T cells.

**Enhancement of T Cell-Mediated Killing of Cancer Cells by SRE Blockade of PD-1/PD-L1**

We hypothesized that SRE induces antitumor responses to blockade PD-1/PD-L1, as T cell activation by medicinal herb extracts has previously been reported (20). To confirm the cytotoxicity of SRE against murine CRC hPD-L1 MC38 cells and hPD-L1 MC38 cell-bearing hPD-1 mice-isolated splenocytes,
Cells were incubated with various concentrations of SRE for 72 hours. SRE had no cytotoxic effect on hPD-L1 MC38 cells up to the concentration of 50 μg/mL (Figure 3A). CC50 concentrations of SRE on these cells were 114.94 ± 14.09 μg/mL. SRE increased splenocyte viability dose-dependently, suggesting that SRE can improve immune cell function (Figure 3B).

To elucidate whether T cells mediate SRE’s antitumor effect, coculture systems were conducted; hPD-1 splenocytes were used as effector cells, and hPD-L1 MC38 cancer cells known to express PD-L1 were used as target cells (21). At an effector cell-to-target cell ratio of 10:1, the cytotoxicity of hPD-L1 MC38 cells cocultured with hPD-1 splenocytes was gradually increased concentration-dependently (Figures 3C, D). CC50 concentrations of SRE on the hPD-L1 MC38 cells for 72 hours were 33.25 ± 7.20 μg/mL (Figure 3C). Moreover, released IL-2 increased dose-dependently, suggesting that T cells activated by SRE secrete IL-2 (Figure 3E). These results imply that SRE efficiently improved T cell immune function by blocking the PD-1/PD-L1 immune checkpoint pathway.
SRE Activation of hPD-1+ Tumor-Infiltrating CD8+ T Cells From hPD-L1 MC38 Tumor Tissues

We confirmed that SRE suppressed hPD-L1 MC38 tumor growth in both in vitro and in vivo models by activating hPD-1 T cells. The additional delineation of the anticancer effect of tumor-infiltrating CD8+ T cells based on T cell activation by SRE treatment in CRC can also be targeted by immunotherapy. To further examine the cytotoxic role of tumor-infiltrating CD8+ T cells by SRE treatment, coculture systems were conducted using hPD-L1 MC38 cell-bearing hPD-1 knockin mice. Murine CRC hPD-L1 MC38 cells and hPD-1 mice splenocytes were treated with SRE for 72 hours. (C) Cocultured hPD-L1 MC38 cell viability tested by crystal violet staining; (D) Lactate dehydrogenase (LDH) release from the hPD-L1 MC38 cells, detected with use of LDH cytotoxicity assay; (E) Relative interleukin-2 (IL-2) level, determined using the mouse IL-2 ELISA set. Data are presented as the mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the control.

Antitumor Effect of SRE in hPD-L1 MC38 Tumor-Bearing Humanized PD-1 Mouse Model

We examined whether SRE could increase inhibition of tumor growth induced by activated T cells on the hPD-L1 MC38 cell-
bearing humanized PD-1 knockin mouse model. The hPD-L1 MC38 murine CRC cells were injected into the allograft mice model. After 10 days, tumor volumes reached 20 mm$^3$ and mice were randomized into groups of six animals per group to investigate the antitumor effect in vivo, of treatment with SRE and anti-PD-1 antibodies. SRE significantly inhibited hPD-L1 MC38 allograft tumor growth in a dose-dependent manner without affecting body weight, as observed by decreased tumor volume and weight (Figures 5A–E). Notably, the combination of SRE and anti-PD-1 antibodies synergistically suppressed tumor growth to a greater extent than did either agent alone. SRE and anti-PD-1 antibodies did not change aspartate aminotransferase (AST), alanine aminotransferase (ALT), BUN, or creatinine levels in mice serum (Table 1).

Immunohistochemistry staining showed that SRE and anti-PD-1 antibodies increased CD8 (a marker of CD8$^+$ T cells) and PRF1 granule exocytosis involved in cytotoxic T cell-mediated tumor cell death in the tumor tissues (Figure 5F). These investigations demonstrated that the combination of SRE and anti-PD-1 antibodies successfully suppressed tumor growth by improving CD8$^+$ T cell infiltration with antitumor immunity in the humanized PD-1 mouse model.

**DISCUSSION**

Research into antitumor immunity through immune checkpoint blockades with an anti-PD-1/PD-L1 interaction for the treatment of patients with CRC is notable (7). Some CRCs are characterized by advanced/metastatic solid malignancies, TIL enrichment, and upregulated PD-L1 expression within the tumor microenvironment (22). Pembrolizumab, as an anti-PD-1, has been approved for immunotherapy of CRC with microsatellite instability or mismatch-repair deficiency (23). However, clinical agents have a large molecular weight, which is slowly absorbed, and cause various side effects (3). To overcome the shortcomings of
Figure 5 | Sanguisorbae Radix extract reduced tumor growth in the hPD-L1 MC38 cell allograft hPD-1 mouse model. (A) Body weight (grams); (B) Tumor volume after 18 days; (C) Tumor weight after 18 days; (D) Images of tumor tissues (bar indicates 5 mm); (E) hPD-L1 MC38 tumor-bearing mice 18 days after treatment; (F) Representative microscopic images (×400) of CD8 and PRF1-positive area of tumor tissues calculated using immunohistochemical analysis. Data are presented as mean ± standard deviation. *p < 0.05, **p < 0.01, and ***p < 0.001 compared with the vehicle group.
TABLE 1 | AST, ALT, BUN, and creatinine levels in SRE-treated mice serum.

|          | AST (IU/L) | ALT (IU/L) | BUN (mg/dL) | Creatinine (mg/dL) |
|----------|------------|------------|-------------|-------------------|
| Vehicle  | 97.66 ± 9.33 | 28.00 ± 9.03 | 32.33 ± 1.41 | 0.1               |
| SRE 100 mpk | 90.00 ± 30.54 | 19.33 ± 3.93 | 28.66 ± 2.50 | 0.1               |
| SRE 300 mpk | 74.00 ± 23.01 | 20.66 ± 4.67 | 35.66 ± 3.75 | 0.1               |
| αPD-1    | 71.33 ± 16.47 | 23.33 ± 5.88 | 35.40 ± 5.60 | 0.1               |
| SRE 300+αPD-1 | 82.00 ± 32.56 | 25.33 ± 8.71 | 30.53 ± 4.78 | 0.1               |

Values are presented as the mean ± SD of six mice. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen.

clinical therapies in the CRC treatment, we tried to use an oriental medicinal herb, SRE, which has a low molecular weight.

Although several reports have shown SRE use in treatment of human CRC, breast cancer, and prostate cancer (18, 24, 25), SRE's anticancer activities via mediation of T cell function by improving T cell activity with SRE. In the present study, we discovered the ability of SRE to enhance T cell functionality via PD-1/PD-L1 interaction blockade using PD-1/PD-L1 ELISA-binding assay and PD-1/PD-L1 blockade bioassay. Additionally, we found a difference, in that SRE activates tumor-infiltrating CD8+ T cells and kills CRC cells in the tumor microenvironment. In particular, this study confirmed that CD8+ T cells activated by SRE secrete PRF1 to kill CRC cells.

Nontoxic dose of SRE in splenocyte-tumor coculture systems, we tested the cytotoxicity of SRE in hPD-L1 MC38 cells and humanized PD-1 mice-isolated splenocytes (Figure 3). In line with a previous study (26), SRE over 100 μg/mL is cytotoxic to a liver cancer cell line; however, 50 μg/mL of SRE has a 40% cytotoxicity and extremely released IL-2 in splenocyte-tumor coculture systems. Interestingly, the more an SRE dose increases up to 400 μg/mL, the more proliferation of splenocytes increases and tumor proliferation decreases. There are reports of SRE inducing immunomodulatory effects including inflammatory responses and cytokine production (27, 28). Our findings suggest that SRE would not only have a cytotoxic effect on CRC cells but also promote T cell immunity and have anticancer effects via inhibition of PD-1/PD-L1 interaction in splenocyte-tumor coculture systems.

The humanized immune checkpoint mice are carefully designed as immuno-oncology mouse models for reliable in vivo evaluation and validation of checkpoint blockers drugs and their combination with other antitumor drugs (29). According to previous studies, although there are structural similarities between human and mouse PD-L1 proteins, there are significant differences in the druggability of these two proteins (30). In line with reported results, our study showed that small molecules, peptides, and some human anti-PD-L1 antibodies bound to human PD-L1, but not to mouse PD-L1. In addition, there were no effects observed in MC38 tumor-bearing immunocompetent mice treated with human anti-PD-L1 antibodies. Moreover, the MC38 used in vivo in this study is derived from tumors induced by carcinogen and established in C57BL/6 mice and represents a microsatellite-unstable CRC cell line (31). We have previously reported the PD-1/PD-L1-inhibiting abilities of medicinal herbs including Salvia plebeia alone and Rubus coreanus alone in vivo (20, 32). Here, we found that SRE in combination with anti-PD-1 antibodies in vivo is a potent PD-1/PD-L1 inhibitor. We assessed the synergistic effect of the combination of SRE and anti-PD-1 antibodies using humanized PD-1 knockin mice and humanized PD-L1 MC38 tumor cells and successfully established a CRC immunotherapy. As shown in Figure 5, the antitumor effect of SRE was higher in a dose of 300 mg/kg group than in a 100 mg/kg group; the effect of SRE in combination with anti-PD-1 antibodies was considerably greater than 300 mg/kg SRE alone or anti-PD-1 antibodies alone. In addition, combination of SRE and anti-PD-1 antibodies remarkably increased infiltration of CD8+ T cells in tumor tissues more than either 300 mg/kg SRE alone or anti-PD-1 antibodies alone, as well as increasingly released PRF1 granules of tumor-infiltrating CD8+ T cells via PD-1/PD-L1 blockade in the tumor microenvironment. The combination of SRE and anti-PD-1 antibodies is thus expected to be a workhorse for preclinical investigational studies in patients with CRC, supporting the validity of cancer immunotherapies.

Biochemical analysis also confirmed that the indicated dosages of SRE and anti-PD-1 antibodies have no significant toxic effect on the liver or kidneys in immunized PD-1 mice with humanized PD-L1 MC38 tumors (Table 1). In a Mongolian gerbil, daily oral administration of SRE at a dose of 400 mg/kg for 28 days, no gross histological changes were found (33). Moreover, the no changes were found in the relative weights of liver and kidney in the gerbil, or in AST and ALT in high-fat–diet-induced obese C57BL/6J mice after 8 weeks’ treatment with 200 mg/kg/day SRE extracted with 50% ethanol (34). In the current study, 100 mg/kg/day of SRE in mice is equivalent to 480 mg/kg/day for a 60 kg human. S. officinalis is safe enough to be approved for use as a food ingredient, and the root of the plant, SR, is considered nontoxic.

This study provides evidence that SRE enhanced the potential antitumor immunologic response by regulating the PD-1/PD-L1 axis for the treatment of CRC. Additionally, it established that SRE in combination with anti-PD-1 antibodies has strong CD8+ T cell-mediated antitumor activity in an hPD-L1 MC38 cell-bearing hPD-L1 knockin mouse model. Recently, because ICIs alone have shown limited efficacy in patients with CRC, combinations of ICIs with other agents, such as anti-PD-L1 antibodies or chemotherapy, are being tested in patients with CRC in clinical studies (10); however, applied combination therapies have several adverse effects, including toxicity, resistance, and side effects (3). To improve upon the clinical combination therapies, we used a combination of clinical antibodies with SRE, with easy dose adjustment and fast absorption in a humanized mice model.

Among the ingredient compounds of SR, several phenolic compounds have been reported as antitumor agents (19). Several other chemical constituents, including tannins, triterpenoids, flavonoids, and triterpene glycosides, have been found in S.
officinalis roots (35–37). It is not clear which components are responsible for PD-1/PD-L1 blockade and the subsequent antitumor effect of CRC. Further studies are needed to elucidate, which molecules from SR inhibit the PD-1/PD-L1 interaction.

CONCLUSIONS

We demonstrated that SRE inhibits PD-1/PD-L1 interaction and suppresses growth of CRC cells by enhancing T cell functional activity. In addition, we established that SRE in combination with anti-PD-1 antibodies significantly reduces CRC tumor growth via tumor-infiltrating CD8+ T cell activities in an hPD-L1 MC38 cell-bearing hPD-1 knockin mouse model. From these results, we suggest that SRE is a novel inhibitor of PD-1/PD-L1 interaction and, in combination with antibody drugs, may provide a new strategy for weakening CRC mortality as an antitumor immunity drug.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

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ETHICS STATEMENT

The animal study was conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Oriental Medicine (KIOM), and approved by the IACUC of the KIOM (approval number: KIOM-D-20-073).

AUTHOR CONTRIBUTIONS

E-JL, J-GC, and H-SC designed the study, conducted the experiments and wrote the manuscript. JK, TK, Y-JK, MP, CJ, YP, WL, and YK performed the experiments. H-SC supervised the research and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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