ORIGINAL PAPER

Nagoya J. Med. Sci. **83**. 683–696, 2021
doi:10.18999/nagjms.83.4.683

S-1 facilitates canerpaturev (C-REV)-induced antitumor efficacy in a triple-negative breast cancer model

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ABSTRACT

Canerpaturev (C-REV) is a highly attenuated, replication-competent, mutant strain of oncolytic herpes simplex virus type 1 that may be an effective new cancer treatment option. S-1, an oral formulation containing the 5-fluorouracil (5-FU) prodrug tegafur and the two enzyme modulators gimeracil and oteracil, is used as a key chemotherapeutic agent for metastatic recurrent breast cancer. Although the antitumor effects of oncolytic viruses combined with 5-FU in vivo have been reported, the detailed mechanisms are unknown. Here, we investigated the antitumor mechanism of the combination of C-REV and S-1 in triple-negative breast cancer (TNBC) in the context of tumor immunity. The combined effect of C-REV and S-1 was evaluated in a bilateral tumor model of murine TNBC 4T1 in vivo. S-1 enhanced the TNBC growth inhibitory effects of C-REV, and decreased the number of tumor-infiltrating, myeloid-derived suppressor cells (MDSCs), which suppress both innate and adaptive immune responses. Moreover, C-REV alone and in combination with S-1 significantly increased the number of $CD8^+$ T cells in the tumor and the production of interferon γ (IFNγ) from these cells. Our findings indicate that C-REV suppresses TNBC tumor growth by inducing the expansion of effector $CD8^+$ T cell subsets in tumors in which S-1 can inhibit MDSC function. Our study suggests that MDSCs may be an important cellular target for breast cancer treatment. The combination of C-REV and S-1 is a new approach that might be directly translated into future clinical trials against TNBC.

Keywords: oncolytic virus, canerpaturev, S-1, myeloid-derived suppressor cells

Abbreviations:
C-REV: canerpaturev
TNBC: triple-negative breast cancer
MDSC: myeloid-derived suppressor cell
IFNγ: interferon γ
HER2: epidermal growth factor 2
5-FU: 5-fluorouracil

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OV: oncolytic virus
MOI: multiplicity of infection
TAA: tumor-associated antigen
DC: dendritic cell
TDLN: tumor-draining lymph node

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INTRODUCTION

Although breast cancer is the most common cancer in women in Japan, breast cancer with distant metastasis is difficult to cure by excision and requires systemic treatment with various drugs.[1-3] Triple-negative breast cancer (TNBC) is hormone receptor negative (estrogen receptor (ER) and progesterone receptor (PR) negative), as well as human epidermal growth factor 2 (HER2) negative.[4] Hormonal therapy is effective against luminal types with ER- or PR-positive expression, while anti-HER2 therapy, such as trastuzumab, is effective against HER2-positive breast cancer. Of approximately one million new breast cancer cases diagnosed annually worldwide, approximately 170,000 (12%–20%) are TNBC.[5] TNBC is not susceptible to either hormonal therapy or anti-HER2 therapy, and chemotherapy is therefore the standard treatment. Due to the high incidence of TNBC and the lack of effective treatments, there is a significant need to identify new therapies for this condition.

5-fluorouracil (5-FU) has been used to treat diverse cancer types and is accepted worldwide as a therapy for breast cancers.[6] S-1 is an oral combination of tegafur, gimeracil (a potent dihydropyrimidine dehydrogenase inhibitor), and oteracil potassium (an inhibitor of phosphorylation of 5-FU in the gastrointestinal tract).[7] As tegafur is a prodrug of 5-FU, bioactivation of tegafur to 5-FU is catalyzed by CYP2A6 in the liver.[8] S-1 has been approved in Japan for gastric, colorectal, pancreatic, biliary, and head and neck cancers, as well as non-small-cell lung cancer and inoperable or relapsed breast cancer.[9-16]

Viral oncolysis refers to the destruction of a tumor cell following viral infection. The field of oncolytic virotherapy has steadily evolved over the decades, and is now rapidly maturing since many so-called oncolytic viruses (OVs) have found their way into clinical use.[17] OVs exert their antitumor effect through a dual mechanism of action, specifically a direct lytic effect on tumor cells and the induction of anti-cancer adaptive immunity. The efficacy of oncolytic viral therapy for advanced cancer has been shown in preclinical and clinical studies.[18] Canerpaturev (C-REV) is a highly attenuated, replication-competent, mutant strain of HSV-1. Genetically, C-REV naturally lacks the expression of UL43, UL49.5, UL55, UL56, and latency-associated transcripts and overexpresses UL53 and UL54.[19] Lack of UL56 expression may reduce neuroinvasiveness.[20] We have shown that C-REV has potent antitumor activity, both alone and in the context of combination therapy, against preclinical models of melanoma and pancreatic, breast, colon, and bladder cancers.[19] Moreover, the safety and efficacy of C-REV have been demonstrated in phase I and II clinical trials targeting melanoma and pancreatic, breast, and head and neck cancers.[19]

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that expand during the course of chronic inflammation and that co-express $CD11b^+Gr1^+$ in mice and $CD11b^+CD14\text{-}CD33^+$, LIN-HLA-DR-$CD33^+$, or $CD11b^+CD14^+$ in humans.[21,22] MDSCs are found in many cancer patients, including those with breast, head and neck, and non-small cell lung cancers. They accumulate in the blood, lymph nodes, bone marrow, and tumor sites in many human cancers and animal tumor models. MDSCs contribute to the immune tolerance of cancer, notably by inhibiting both adaptive and innate immunity.[23] In addition, they interfere with tumor

immunity and promote tumor growth by inhibiting tumor cell cytotoxicity mediated by natural killer cells,[24] and by blocking the activation of tumor-reactive $CD4^+$ and $CD8^+$ T cells.[25-28] MDSCs are also thought to facilitate immune suppression and tumor progression by inducing the accumulation of immunosuppressive regulatory T cells.[29]

Although the antitumor effects of OVs combined with 5-FU have been reported in vivo,[30,31] the detailed mechanisms are unknown. Here, we investigated the antitumor mechanism of the combination of C-REV and S-1 in the context of tumor immunity. This combination exerted an antitumor effect by inducing $CD8^+$ T cell infiltration into tumors and reducing tumor-infiltrating MDSCs. This suggests that a combined approach involving OV-mediated tumor lysis, induction of $CD8^+$ T cell infiltration into tumors, and reduction of tumor MDSCs might become a new therapeutic strategy against TNBC.

MATERIALS AND METHODS

Drugs

S-1 was purchased from Taiho Pharmaceutical Co., Ltd (Tokyo, Japan). 5-FU was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Cell lines and viruses

The African green monkey kidney cell line Vero was obtained from the American Type Culture Collection (Manassas, VA). The murine breast cancer cell line 4T1 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The Vero and 4T1 cell lines were grown in DMEM (Sigma, Tokyo, Japan) supplemented with 10% FCS and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA). C-REV was provided from Takara Bio Inc (Kusatsu, Japan). C-REV was propagated in Vero cells and stored in aliquots at –80°C. For in vitro and in vivo experiments, C-REV was diluted with phosphate-buffered saline. Viral titers were assayed in Vero cells and expressed as plaque-forming units per milliliter (PFU/mL).

Combined effect of C-REV and 5-FU

Cell viability was analyzed by MTT assay, as previously described,[32] Tumor cells were seeded in 24-well plates (1×10^5 cells/well) and incubated with DMEM at 37°C/5% CO2. After 24 hours, serial dilutions of 5-FU were added and/or cells were infected with C-REV at several multiplicities of infection (MOIs). The first day of treatment was defined as day 0, and cells were grown for 3 days. The number of viable cells was quantified using colorimetric MTT assays. The synergistic reaction was assessed using combination index (CI) values that reflect the potential interactions between C-REV and S-1, where CI >1 indicates a synergistic effect, and CI <1 indicates antagonism.

Animal studies

Female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were kept under constant temperature and humidity conditions and fed with a standard diet and water ad libitum. All experiments were reviewed and approved by the Animal Care University Committee following the Guidelines for Animal Experimentation at Nagoya University (31322, 31323). A bilateral tumor model of 4T1 was used to evaluate antitumor effects. Tumors were dissected and 8 mm^3 tumor pieces were implanted subcutaneously into each flank (right and left). After implantation, tumors were allowed to reach a volume of 100–200 mm^3, and treatments were then started on day 0. The dosing schedule for S-1 followed previous reports.[33] The mice were

treated with 10 mg/kg S-1 for 5 consecutive days per week for 2 weeks by oral gavage. C-REV (5×10^5 PFU/100 μL, i.t.) was injected into the right tumor on day 0, day 2, day 7, and day 9. The body and tumor weights were monitored. The tumor volume was calculated from caliper measurements of tumor length and width, as follows: tumor volume = $1/2 \times a \times b^2$, where a represents the length and b represents the width (mm). For the evaluation of survival, death event was defined when the total tumor size reached 1500 mm^3.

Tumor disaggregation and re-stimulation of tumor-infiltrating lymphocytes in vitro

4T1 tumors were dissociated into single-cell suspensions using the MACS murine tumor dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, tumors from drug-treated mice were cut into 3 mm fragments and transferred into a C-tube (Miltenyi Biotec) with Enzyme A, Enzyme D, and Enzyme R in the kit. The samples were placed onto the GentleMACS dissociator according to the manufacturer's instructions. Following disaggregation, the cell suspension was passed through a 70-μm strainer and centrifuged at 400 × g for 5 minutes. Tumor-infiltrating lymphocytes were restimulated as described.[34] Briefly, cells were labeled using a Miltenyi CD8α T cell enrichment kit (Miltenyi Biotec) and isolated using magnetic sorting according to the manufacturer's protocols. Tissue culture plates were coated with 5 μg/mL anti-CD3 antibody (145-2C11; BioLegend, San Diego, CA) in PBS for 12 hours, and excess antibody was aspirated before T cell addition. Cells were cultured for 72 hours before the addition of 2 μM monensin for 4 hours for intracellular interferon γ (IFNγ) staining.

Antibodies and flow cytometry

Single-cell suspensions obtained from mouse tumors were analyzed using a FACS Calibur or Canto II flow cytometer (BD Biosciences, San Jose, CA). Cells were first treated with anti-CD16/32 (93) for 10 minutes at 4°C, then with specifically conjugated antibodies for 30 minutes at 4°C in the dark. The following anti-mouse antibodies were used in the analysis: CD45 (30F-11), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), IFNγ (XMG1.2), and Gr-1 (1A8-Ly6g). All antibodies were purchased from BioLegend, Thermo Fisher Scientific, or BD Biosciences. These antibodies were conjugated to the following fluorescent dyes: fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein–cyanine 5.5, and allophycocyanin. FlowJo software (BD Biosciences, version 10.6) was used to analyze flow cytometric data.

Statistical analysis

Continuous variables were analyzed by ANOVA with a post-hoc Tukey test. Survival analysis was performed using the Kaplan–Meier method. The log-rank test was used for statistical comparison of the curves. Differences were considered statistically significant when p-values were less than 0.05. Analyses were conducted by Prism 7 software (GraphPad Software, La Jolla, CA) and IBM SPSS statistics (IBM, Chicago, IL)

RESULTS

The combination of C-REV and S-1 treatment leads to stronger regression of murine 4T1 tumors

The sensitivities of the murine TNBC 4T1 cell line to C-REV, 5-FU, and their combination were evaluated by MTT assays. 5-FU was used in in vitro experiments as a substitute for S-1 since S-1 is composed of tegafur, a prodrug of 5-FU. 5-FU inhibited 4T1 cell growth in a concentration-dependent manner. When C-REV (MOI 0.01) was added to 5FU (1 mM), synergistic cytotoxicity was observed in vitro (Figure 1a and Table 1). We next assessed the therapeutic effects of the combination treatment in a bilateral 4T1 tumor model in which mice were subcutaneously inoculated in both flanks. C-REV was injected into the tumor on only one side (referred to as the injected side; the non-injected side is the contralateral side), and tumor sizes on both sides were measured twice a week. When tumors reached between 100 and 200 mm^3, S-1 was given to mice p.o. at a dose of 10 mg/kg for 5 consecutive days per week for 2 weeks. C-REV (5×10^5 PFU/100 μL, i.t.) was administered to mice on day 0, day 2, day 7, and day 9. C-REV, S-1, and their combination led to tumor regression and prolonged survival (Figure 1b, c). The combination of C-REV and S-1 was markedly more effective than C-REV alone against non-injected tumors, and prolonged survival in vivo compared to either agent alone. Overall, the combination of C-REV and S-1 treatment led to regression of both injected and non-injected tumors.

Table 1 Fractional cell survival (FCS) of 4T1 tumor cells following treatment with 5-FU and C-REV alone or in combination

Conc. of 5-FU (mM)	**5-FU**	**C-REV (MOI 0.01)**	**C-REV (MOI 0.01) + 5-FU**		**Combination index (CI) (E-FCS/O-FCS)**
			E-FCS	O-ECS	
0.01	0.352	0.934	0.329	0.370	0.891
0.1	0.176	0.934	0.164	0.166	0.990
1	0.126	0.934	0.118	0.104	1.138*

FCS: fractional cell survival (mean cell survival experimental/mean cell survival control)
E-FCS: expected FCS (mean FCS for C-REV × mean FCS for 5-FU)
O-FCS: observed FCS
CI > 1 indicates synergic effect

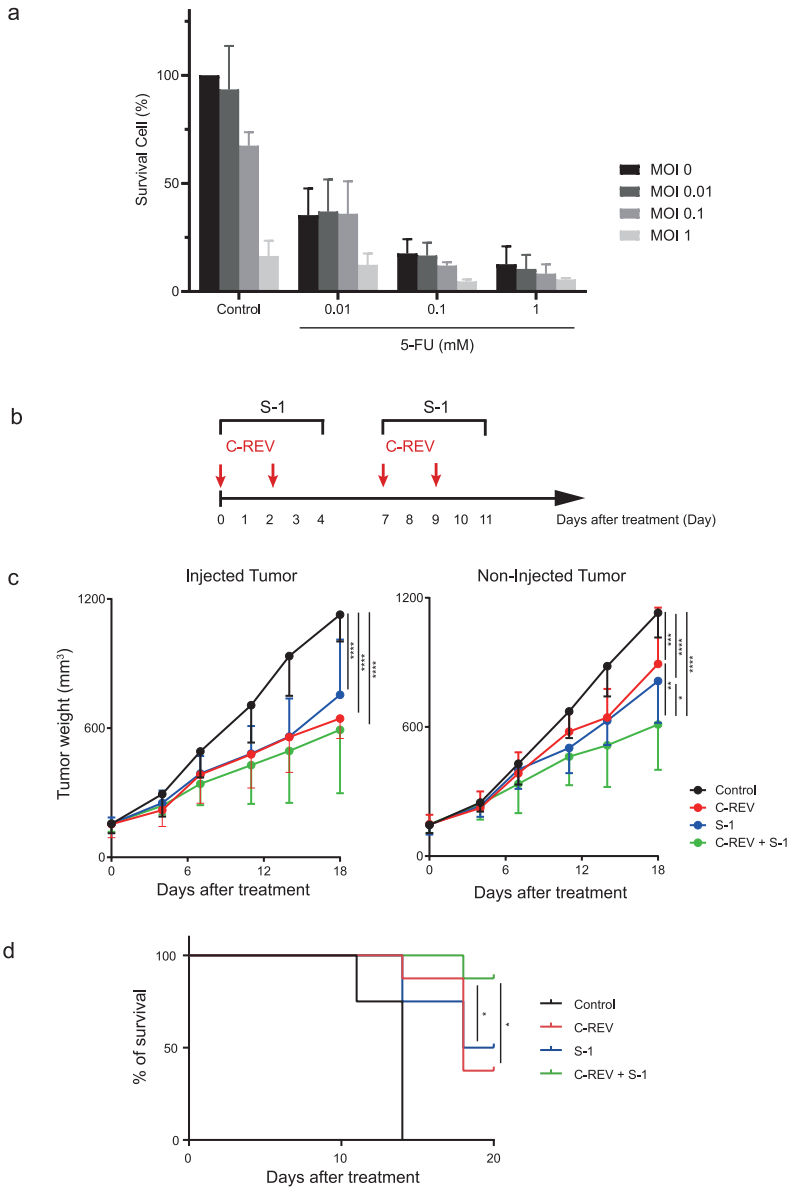

Fig. 1 The combination of C-REV and S-1 treatment leads to stronger regression of murine 4T1 tumor

Fig. 1a: The in vitro sensitivity of 4T1 cells to C-REV with or without 5-FU was determined by MTT assays. Cells were infected with C-REV at various multiplicities of infection (MOIs). The results are shown as means ± SD.

Fig. 1b and 1c: Female 6- to 7-week-old Balb/c mice were inoculated with 4T1 tumor in the right and left flanks. When the average tumor volumes reached 100–200 mm^3, mice were randomly divided into groups (n=8 per group) with equal average tumor volumes among both groups and both flanks. Fig. 1b, Treatment schema: the mice were treated with 10 mg/kg S-1 for 5 consecutive days per week for 2 weeks. C-REV was administered i.t. to mice on day 0, day 2, day 7, and day 9. Fig. 1c, C-REV was injected into the tumor on only one side (referred to as the injected side; the non-injected side is the contralateral side), and tumor sizes on both sides were measured twice a week.

Fig. 1c: Tumor growth and **Fig. 1d:** mouse survival in 4T1 tumor models after treatment. For the evaluation of survival, death event was defined when the total tumor size reached 1500 mm^3. ANOVA with a post-hoc Tukey test was performed for tumor size evaluation. Survival analysis was performed using the Kaplan–Meier method. The log-rank test was used for statistical comparison of the curves.

* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$

C-REV enhances the tumor accumulation of CD3⁺CD8⁺ T cells and their production of IFNγ

OVs selectively replicate within cancer cells and then kill them without harming normal tissues. In addition to this direct oncolytic activity, OVs evoke an immune response by releasing tumor- and pathogen-associated antigens that enhance antigen presentation and antitumor immunity.[35,36] We next investigated the infiltration of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells into tumors. Mice were given both S-1 (10 mg/kg) by oral gavage (5 consecutive days, days 0–4) and intratumoral injections of C-REV (5×10^5 PFU) on day 0 and day 2 into the right tumor. Three days after final drug treatment, tumors were extracted and tumor-infiltrating lymphocytes were isolated. We found that C-REV significantly increased the numbers of $CD3^+CD8^+$ T cells in both injected and non-injected tumors (Figure 2b). However, no increase in $CD3^+CD4^+$ T cells was demonstrated (Figure 2a). We further tested whether IFNγ was induced in tumor-infiltrating $CD3^+CD8^+$ T cells after treatment with C-REV, S-1, and the combination of both. We isolated these cells from 4T1 tumors after drug treatment and subjected them to anti-CD3/anti-CD28 antibody stimulation. Stimulated $CD3^+CD8^+$ T cells from injected tumors showed significantly increased IFNγ production compared to stimulated control $CD3^+CD8^+$ T cells (Figure 2c). Furthermore, enhanced IFNγ production was also observed in stimulated $CD3^+CD8^+$ T cells derived from non-injected tumors (Figure 2c). These data demonstrated that C-REV induced infiltration of $CD3^+CD8^+$ T cells into tumors and that C-REV, S-1, and their combination all increased IFNγ production by $CD3^+CD8^+$ T cells.

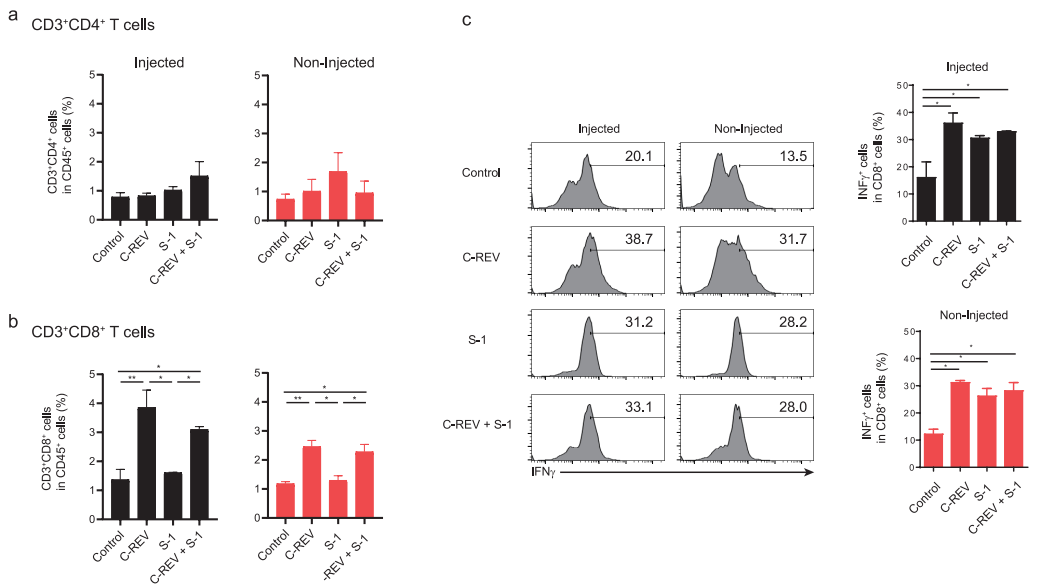

Fig. 2 C-REV enhances the infiltration of $CD3^+CD8^+$ T cells into tumors and increases IFNγ production by $CD3^+CD8^+$ T cells

Female 6- to 7-week-old Balb/c mice were inoculated with 4T1 tumor in the right and left flanks. When the average tumor volumes reached 100–200 mm^3, mice were randomly divided into groups with an equal average tumor volume among the groups and both flanks. Each group contained three mice. Mice were given S-1 (10 mg/kg) by oral gavage (5 consecutive days, day 0–4) in addition to an intratumoral injection of C-REV (5×10^5 PFU) on day 0 and day 2. Tumors were harvested 3 days after the final treatment from both the injected and non-injected sites.

Fig. 2a: $CD4^+$ T cells and **Fig. 2b:** $CD8^+$ T cells, gated from $CD45^+CD3^+$ cells after treatment with C-REV, S-1, and their combination, were analyzed by flow cytometry.

Fig. 2c: Tumor-infiltrating lymphocytes were isolated. These cells were stimulated with anti-CD3/anti-CD28 antibody, and intracellular staining of IFNγ was performed.

* $p < 0.05$, * * $p < 0.01$

S-1 depletes splenic and tumor MDSCs, and C-REV decreases tumor MDSCs

It was previously shown that 5-FU reduced the numbers of splenic and tumor MDSCs in cancer-bearing hosts.[37] MDSCs have been identified as a population of immature myeloid cells with the ability to suppress T cell activation in humans and mice.[38] Furthermore, 4T1 tumor progression is associated with the accumulation of MDSCs.[27] Based on this knowledge and the fact that $CD3^+CD8^+$ T cells enhanced IFNγ production after treatment with C-REV, S-1, and their combination, we hypothesized that the ability of S-1 to reduce the numbers of splenic and tumor MDSCs would enhance the antitumor activity of C-REV. To examine this possibility, MDSC numbers were measured in 4T1 tumor-bearing mice after drug treatment. As in previous reports, treatment with S-1 dramatically reduced splenic enlargement (Figure 3a) and the number of splenic MDSCs ($CD11b^+Gr\text{-}1^+$), whereas C-REV did not decrease the number of splenic MDSCs (Figure 3b, c). In addition, C-REV, S-1, and their combination all significantly decreased the number of MDSCs in both injected and non-injected tumors (Figure 4). These results suggest that S-1 is able to reduce the numbers of splenic and tumor MDSCs, while C-REV suppresses tumor MDSCs.

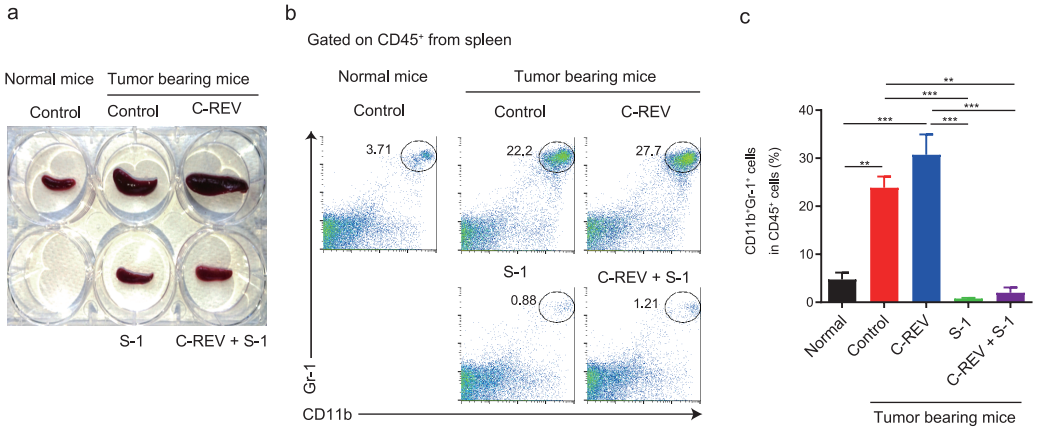

Fig. 3 S-1 and the combination of S-1 and C-REV eliminate MDSCs in the spleen in vivo

Fig. 3a: Mice were given S-1 (10 mg/kg) by oral gavage (5 consecutive days, day 0–4) in addition to an intratumoral injection of C-REV (5×10^5 PFU) on day 0 and day 2. Three days after the final drug treatment, spleens were harvested, and MDSC infiltration was determined by FACS analysis (n=3 per group).

Fig. 3a: Representative spleens from the different experimental groups are presented.

Fig. 3b The percentage of cells that were MDSCs ($CD11b^+Gr1^+$) was determined after treatment by FACS analysis.

Fig. 3c: The graph shows the percentages of splenic MDSCs.

* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$

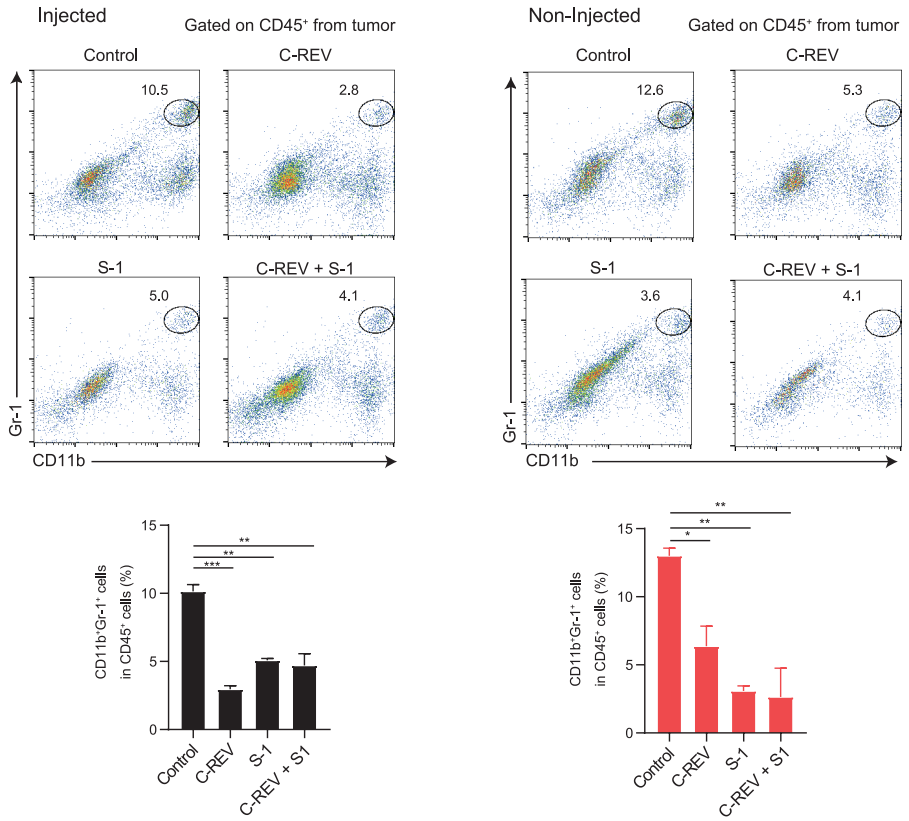

Fig. 4 Treatment with C-REV and S-1 reduces tumor MDSC infiltration
Mice were given S-1 (10 mg/kg) by oral gavage (5 consecutive days, days 0–4) in addition to an intratumoral injection of C-REV (5×10^5 PFU) on day 0 and day 2. Three days after final drug treatment, tumors were extracted from mice, and tumor-infiltrating lymphocytes were isolated. MDSC infiltration was determined by FACS analysis (n=3 per group). Graphs show the percentages of tumor MDSCs.
* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$

C-REV and the combination of C-REV and S-1 increase IFNγ production by T cells in tumor-draining lymph nodes (TDLNs)

Although C-REV did not decrease the number of splenic MDSCs, it significantly reduced the number of MDSCs in both injected and non-injected tumors, and also inhibited tumor growth. OVs induce tumor cell lysis and release tumor-associated antigens (TAAs) that not only enhance antigen presentation, but also induce pathogen- and danger-associated molecular patterns that further augment antigen presentation and activation of dendritic cells (DCs).[35,36] DCs transport tumor and virus antigens to TDLNs and cross-present antigens to activate $CD3^+CD8^+$ T cells. Thus, an effective anti-tumor response requires $CD3^+CD8^+$ T cell activation that depends on tumor antigen presentation by DCs in TDLNs. We hypothesized that after C-REV treatment, DCs that capture TAAs could activate $CD3^+CD8^+$ T cells in TDLNs. To verify this, we evaluated the number of MDSCs and T cells in TDLNs, as well as IFNγ production by isolated $CD3^+CD8^+$ T cells in TDLNs. While all groups (C-REV, S-1, their combination, and controls) demonstrated few MDSCs in TDLNs (less than 0.2%), S-1 treatment significantly reduced their number (Figure 5a). In contrast, no treatment affected T cell populations in TDLNs (Figure 5b). Next, we examined

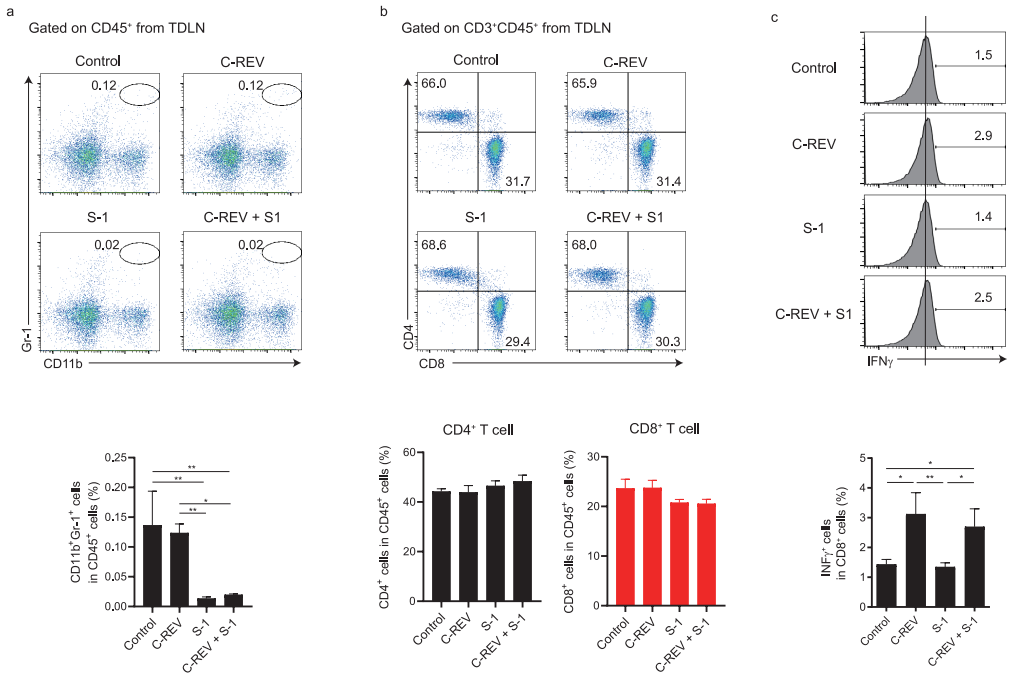

Fig. 5 C-REV enhances IFNγ production by $CD3^+CD8^+$ T cells in tumor-draining lymph nodes

Female 6- to 7-week-old Balb/c mice were inoculated with 4T1 tumor in the right and left flanks. When the average tumor volumes reached 100–200 mm^3, mice were randomly divided into groups (n=3/group) with an equal average tumor volume among the groups and both flanks. Lymphocytes were harvested 3 days after the final treatment from tumor-draining lymph nodes.

Fig. 5a: MDSC gated from $CD45^+$ cells and **Fig. 5b:** T cells and those gated from $CD45^+CD3^+$ cells were analyzed by flow cytometry after treatment with C-REV, S-1, and their combination.

Fig. 5c: T cells isolated from tumor-draining lymph nodes were stimulated with anti-CD3/anti-CD28 antibody, and intracellular staining of IFNγ was performed.

* $p < 0.05$, * * $p < 0.01$

IFNγ production by isolated $CD3^+CD8^+$ T cells in TDLNs. While C-REV and the combination of C-REV and S-1 enhanced the production of IFNγ by $CD3^+CD8^+$ T cells, S-1 alone did not (Figure 5c). These results suggest that the treatment of tumors with C-REV activates $CD3^+CD8^+$ T cells in TDLNs and that the activated $CD3^+CD8^+$ T cells attack tumors regardless of whether they were injected with C-REV, although the reduction of splenic and tumor MDSCs by S-1 is necessary for enhancement of the C-REV antitumor effect.

DISCUSSION

In this study, we performed in vitro and in vivo evaluations of the effect of combining C-REV with S-1 in the treatment of TNBC. Treatment with both C-REV (MOI 0.01) and 5FU (1 mM) had a synergistic effect in terms of cell growth repression in vitro, and resulted in the regression of both injected and non-injected tumors in vivo and prolonged survival compared to either agent alone. The number of MDSCs in tumors decreased after C-REV treatment, and the number of $CD3^+CD8^+$ T cells, which play important anti-tumor roles, was significantly

increased. Furthermore, S-1 alone and the combination of C-REV and S-1 effectively reduced the numbers of splenic and tumor MDSCs, thus reversing the immunosuppressive state of the tumor microenvironment. Collectively, these findings indicate that the combination of C-REV with S-1 may mobilize systemic antitumor immunity in mice.

Although TNBC is highly malignant, recurs quickly, and is often difficult to treat, the development of effective chemotherapy and molecular targeted drugs has progressed in recent years, and further treatment advances are expected. Oral fluoropyrimidines (S-1 and capecitabine) are used for the treatment of metastatic breast cancer to avoid severe adverse effects, although firm supporting evidence is lacking. S-1 is an oral fluorouracil formulation that consists of tegafur, gimeracil, and oteracil potassium in a molar ratio of 1 : 0.4 : 1.[39] Takashima et al reported that as a first-line treatment for patients with metastatic breast cancer, S-1 was non-inferior to taxane with respect to overall survival and better than taxane with regard to health-related quality of life.[12] Thus, S-1 should be considered as a new first-line chemotherapy option for patients with TNBC. In this study, combination therapy with C-REV and S-1 in vivo demonstrated a stronger antitumor effect against murine TNBC tumors than either agent alone, suggesting that this treatment may be a promising approach that could be directly translated into a future clinical trial against TNBC.

We showed that S-1 effectively reduced the numbers of splenic and tumor MDSCs, and that this decrease may therefore enhance the antitumor effect of C-REV. MDSCs have been identified as immature myeloid cells with the ability to suppress T cell activation in humans and mice by promoting the production of transforming growth factor-β, interleukin-10, reactive oxygen species, nitric oxide, and arginase.[40-44] They are induced by a variety of factors, including vascular endothelial growth factor, granulocyte-macrophage colony-stimulating factor, and proinflammatory cytokines such as interleukin-1β.[45-49] Consistent with our results, Vincent et al reported that 5-FU was able to significantly decrease the numbers of both splenic and tumor MDSCs.[37] S-1 is metabolized to 5-FU, the latter of which depletes MDSCs and selectively induces their apoptosis both in vitro and in vivo.[37] Furthermore, C-REV infection depletes tumor MDSCs and suppresses their function, but the mechanisms of C-REV are unknown and additional studies are needed.

Regarding the mechanism underlying the strong antitumor effect of C-REV and S-1, we considered that in addition to tumor lysis by C-REV and inhibition of DNA synthesis by S-1, C-REV induces the infiltration of $CD3^+CD8^+$ T cells into tumors, and S-1 and C-REV reduce the number of MDSCs, which relieves $CD3^+CD8^+$ T cells suppression and enhances the antitumor effect. To confirm the activity of $CD3^+CD8^+$ T cells in tumors and TDLNs, we measured the production of IFNγ by $CD3^+CD8^+$ T cells. C-REV, S-1, and their combination significantly increased IFNγ levels compared with controls. $CD3^+CD8^+$ T cells may target both viral and tumor antigens, and although our study did not characterize their precise antigen specificity, we previously reported that C-REV treatment increased the cytotoxic activity of lymphocytes with high IFNγ expression that recognized tumor-specific antigens.[50] Our results suggest that the high production of IFNγ by $CD3^+CD8^+$ T cells in tumors and TDLNs results from the combination of the cytotoxic effects of S-1 against MDSCs and the induction of $CD3^+CD8^+$ T cell infiltration into tumors by C-REV.

The fact that C-REV suppressed tumor growth even in non-injected tumors indicates that it may be effective against advanced TNBC with multiple metastases. In addition, it has been reported that MDSCs are metabolically active and that they secrete large quantities of inflammatory cytokines and chemokines that can cause cachexia.[51] Thus the combination of C-REV and S-1 is also expected to prevent cachexia by reducing the numbers of splenic and tumor MDSCs.[51]

Our results show for the first time that the antitumor effects of C-REV, an OV, were enhanced by S-1, and that together they eliminated MDSCs in a mouse TNBC tumor model, resulting in

tumor regression. We also observed that S-1–mediated MDSC depletion triggered an increase in IFNγ production by $CD3^+CD8^+$ T cells that infiltrated tumors as a result of C-REV, and that this process promoted a T cell mediated antitumor effect. In summary, our study suggests that the antitumor immunostimulatory effect of C-REV and the reduction of tumor MDSCs by S-1, as well as C-REV's oncolytic activity and S-1's cytotoxic effects, may constitute a new therapeutic strategy against TNBC. Further studies should elucidate the mechanism whereby C-REV causes $CD3^+CD8^+$ T cell expansion in tumors and devise strategies for maximizing treatment efficacy against metastatic TNBC by combining C-REV with S-1.

AUTHOR CONTRIBUTIONS

N. Miyajima and I.R.E. contributed equally to this work.

ACKNOWLEDGMENTS

This study was supported in part by a Japan Grants-in-Aid for Scientific Research (16H05413 and 16K15611). The authors are thankful to Yukihiro Nishiyama for the isolation of C-REV at the Department of Virology, Nagoya University Graduate School of Medicine. We thank Emi Uematsu and Yumiko Samizo for their excellent technical assistance.

FOOTNOTE

This manuscript has been edited for English language, grammar, punctuation and spelling by ZENIS Co., Ltd. (www.zenis.co.jp).

CONFLICT OF INTERESTS

M.T. is an employee of Takara Bio Inc. The other authors declare no competing interests. The funding sponsor (Takara Bio, Inc.) had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish this paper.

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