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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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-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, and by blocking the activation of tumor-reactive CD4+ and CD8+ T cells. MDSCs are also thought to facilitate immune suppression and tumor progression by inducing the accumulation of immunosuppressive regulatory T cells.

Although the antitumor effects of OVs combined with 5-FU have been reported in vivo, 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. Tumor cells were seeded in 24-well plates (1 x 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 mm³ tumor pieces were implanted subcutaneously into each flank (right and left). After implantation, tumors were allowed to reach a volume of 100–200 mm³, and treatments were then started on day 0. The dosing schedule for S-1 followed previous reports. 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 × 10⁵ 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 × a × b², 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 mm³.

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 mm³, 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 × 10⁵ 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) |
|-------------------|------|-----------------|-------------------------|-------------------------------------|
| 0.01              | 0.352| 0.934           | 0.329                   | 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 mm³, 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 survival in 4T1 tumor models after treatment. For the evaluation of survival, death event was defined when the total tumor size reached 1500 mm³. 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 × 10⁶ 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 mm³, 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 × 10⁶ 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. MDSCs have been identified as a population of immature myeloid cells with the ability to suppress T cell activation in humans and mice. Furthermore, 4T1 tumor progression is associated with the accumulation of MDSCs. Based on this knowledge and the fact that CD3\(^+\)CD8\(^+\) T cells enhanced IFN\(\gamma\) 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-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.

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**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 \(\times\) 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.

\(\ast\) p < 0.05, \(\ast\ast\) p < 0.01, \(\ast\ast\ast\) p < 0.001
S-1 enhances antitumor effect of C-REV

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). 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...
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**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
S-1 enhances antitumor effect of C-REV

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. 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. 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. 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. Consistent with our results, Vincent et al reported that 5-FU was able to significantly decrease the numbers of both splenic and tumor MDSCs. S-1 is metabolized to 5-FU, the latter of which depletes MDSCs and selectively induces their apoptosis both in vitro and in vivo. 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. 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. Thus the combination of C-REV and S-1 is also expected to prevent cachexia by reducing the numbers of splenic and tumor MDSCs.

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.

AUTHOR CONTRIBUTIONS

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

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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.

REFERENCES

1 Gennari A, Conte P, Rosso R, Orlandini C, Bruzzi P. Survival of metastatic breast carcinoma patients over a 20-year period: a retrospective analysis based on individual patient data from six consecutive studies. Cancer. 2005;104(8):1742–1750.
2 Greenberg PA, Hortobagyi GN, Smith TL, Ziegler LD, Frye DK, Buzdar AU. Long-term follow-up of patients with complete remission following combination chemotherapy for metastatic breast cancer. J Clin Oncol. 1996;14(8):2197–2205.
3 Rahman ZU, Frye DK, Smith TL, et al. Results and long term follow-up for 1581 patients with metastatic breast carcinoma treated with standard dose doxorubicin-containing chemotherapy: a reference. Cancer. 1999;85(1):104–111.
4 Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98(19):10869–10874.
5 Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast
S-1 enhances antitumor effect of C-REV.
30 Nakano K, Todo T, Zhao G, et al. Enhanced efficacy of conditionally replicating herpes simplex virus (G207) combined with 5-fluorouracil and surgical resection in peritoneal cancer dissemination models. J Gene Med. 2005;7(5):638–648.

31 Gutermann A, Mayer E, von Dehn-Rothfelser K, et al. Efficacy of oncolytic herpesvirus NV1020 can be enhanced by combination with chemotherapeutics in colon carcinoma cells. Hum Gene Ther. 2006;17(12):1241–1253.

32 Wu Z, Ichinose T, Naoe Y, et al. Combination of Cetuximab and Oncolytic Virus Canerpaturev Synergistically Inhibits Human Colorectal Cancer Growth. Mol Ther Oncolytics. 2019;13:107–115.

33 Hoang NT, Kadonosono T, Kuchimaru T, Kizaka-Kondoh S. Hypoxia-inducible factor-targeting prodru T3P3 combined with gemcitabine or TS-1 improves pancreatic cancer survival in an orthotopic model. Cancer Sci. 2016;107(8):1151–1158.

34 Garris CS, Arlauckas SP, Kohler RH, et al. Successful Anti-PD-1 Cancer Immunotherapy Requires T Cell-Dendritic Cell Crosstalk Involving the Cytokines IFN-gamma and IL-12. Immunity. 2018;49(6):1148–1161.

e7. doi:10.1016/j.immuni.2018.09.024.

35 Melcher A, Parato K, Rooney CM, Bell JC. Thunder and lightning: immunotherapy and oncolytic viruses collide. Mol Ther. 2011;19(6):1008–1016.

36 Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nat Biotechnol. 2012;30(7):658–670.

37 Vincent J, Mignot G, Chalmin F, et al. 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. Cancer Res. 2010;70(8):3052–3061.

38 Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162–174.

39 Shirasaka T, Nakano K, Takechi T, et al. Antitumor activity of 1 M tegafur-0.4 M 5-chloro-2,4-dihydroxy pyridine-1 M potassium oxonate (S-1) against human colon carcinoma orthotopically implanted into nude rats. Cancer Res. 1996;56(11):2602–2606.

40 Filipazzi P, Valenti R, Huber V, et al. Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol. 2007;25(18):2546–2553.

41 Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. 2003;24(6):302–306.

42 Rodriguez PC, Hernandez CP, Quiceno D, et al. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med. 2005;202(7):931–939.

43 Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Rev Immunol. 2007;7(3):162–174.

44 Kusmartsev S, Gabrilovich DI. Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. J Leukoc Biol. 2003;74(2):186–196.

45 Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med. 1996;2(10):1096–1103.

46 Young MR, Wright MA, Lozano Y, et al. Increased recurrence and metastasis in patients whose primary head and neck squamous cell carcinomas secreted granulocyte-macrophage colony-stimulating factor and contained CD34+ natural suppressor cells. Int J Cancer. 1997;74(1):69–74.

47 Bronte V, Chappell DB, Apolloni E, et al. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. J Immunol. 1999;162(10):5728–5737.

48 Bunt SK, Sinha P, Clements VK, Leips J, Ostrand-Rosenberg S. Inflammation induces myeloid-derived suppressor cells that facilitate tumor progression. J Immunol. 2006;176(1):284–290.

49 Song X, Krelin Y, Dvorkin T, et al. CD11b+Gr-1: immature myeloid cells mediate suppression of T cells in mice bearing tumors of IL-1beta-secreting cells. J Immunol. 2005;175(12):8200–8208.

50 Hotta Y, Kasuya H, Bustos I, et al. Curative effect of HF10 on liver and peritoneal metastasis mediated by host antitumor immunity. Oncolytic Virother. 2017;6:31–38.

51 Cuenca AG, Cuenca AL, Winfield RD, et al. Novel role for tumor-induced expansion of myeloid-derived cells in cancer cachexia. J Immunol. 2014;192(12):6111–6119.