Potentiated antitumor effects of APS001F/5-FC combined with anti-PD-1 antibody in a CT26 syngeneic mouse model

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ABSTRACT

APS001F is a strain of Bifidobacterium longum genetically engineered to express cytosine deaminase that converts 5-fluorocytosine (5-FC) to 5-fluorouracil. In the present study, antitumor effects of APS001F plus 5-FC (APS001F/5-FC) in combination with anti-PD-1 monoclonal antibody were investigated using a CT26 syngeneic mouse model. Both of dosing of APS001F/5-FC before and after anti-PD-1 mAb in the combination dosing exhibited antitumor effects as well as prolonged survival over the nontreated control. The survival rate in the combination therapy significantly increased over the monotherapy with APS001F/5-FC and that with anti-PD-1 mAb. Regulatory T cells among CD4⁺ T cells in tumor decreased in the combination therapy, while the ratio of CD8⁺ T cells was maintained in all groups. Taken these results together, APS001F/5-FC not only demonstrates a direct antitumor activity, but also immunomodulatory effects once localized in the hypoxic region of the tumor, which allows anti-PD-1 mAb to exert potentiated antitumor effects.

Graphical Abstract

Combination therapy of APS001F/5FC and anti-PD-1 antibody demonstrates immunomodulatory effects and potentiated antitumor effect in the hypoxic solid tumor.

Keywords: APS001F, anti-PD-1 antibody, Treg, immuno oncology, combination therapy
Programmed cell death 1 (PD-1) is a key immune checkpoint molecule responsible for the immune evasion from the antitumor immune system (Zuazo et al. 2017). Monoclonal antibodies (mAbs) to PD-1 have shown efficacy for various types of cancers as the mainstay of the cancer treatment (Conway et al. 2018; Wu et al. 2019). However, efficacy against gastrointestinal cancer, such as colorectal cancer, is limited (Boland and Ma 2017; Conway et al. 2018; Dosset et al. 2018; Oliveira et al. 2019).

The combination of anti-PD-1 mAb with chemotherapeutic agents is being investigated to improve therapeutic effects in clinical trials (Cully 2015; Tang et al. 2018; Heinhuus et al. 2019). Recent studies suggest that chemotherapeutic agents, including 5-fluorouracil (5-FU), play an immunomodulatory role in the tumor. In a CT26 (mouse colorectal cancer) tumor model, 1 cycle (3 doses) of 5-FU systemic dosing promoted antitumor immune responses by increasing CD8⁺ T cells, but repeated cycles of 5-FU dosing did not. In addition, 1 cycle of 5-FU dosing increased antitumor responses when combined with antiprogrammed cell death-ligand 1 (PD-L1) mAb (Wu et al. 2016).

CT26 syngeneic mouse model is CT26 (colorectal cancer from BALB/c mouse) tumor bearing BALB/c mouse. The model has been widely used to investigate the effect of anti-PD-1 mAb and/or cancer chemotherapeutic agents. Therefore, the model is appropriate to evaluate research results internationally.

It is well known that the hypoxic microenvironment in the tumor is associated with tumor progression, and resistance to radiation therapy and chemotherapy (Brown 1999; Harada 2016; Jing et al. 2019). By taking advantage of these conventionally troublesome anaerobic conditions in tumor tissues, we have been developing a delivery system with nonpathogenic anaerobic bacteria, Bifidobacterium, derived from the human intestine to target solid tumors. It has been confirmed that Bifidobacterium longum (B. longum) of obligate anaerobe selectively localized in hypoxic region in solid tumors and disappeared from normoxic normal tissues within a few days following intravenous (i.v.) injection in a mouse model (Yazawa et al. 2000).

We developed a recombinant B. longum genetically engineered to express cytosine deaminase that converts 5-fluorocytosine (5-FC) to 5-FU (Nakamura et al. 2002). This prototype of APS001F localized and produced 5-FU in tumor tissues in combination with oral 5-FC (Sasaki et al. 1996; Taniguchi et al. 2010). APS001F carries a shuttle-plasmid that includes the mutated cytosine deaminase gene with a mutation at the active site to increase the enzyme activity (patent WO2009/128272, https://patentscope2.wipo.int/search/en/detail.jsf?docId=WO2009128272) than the prototype and currently in phase 1/2 clinical trial in USA (NCT01562626).

In the present study, we investigated the combination effect of APS001F/5-FC with anti-PD-1 mAb in a CT26 syngeneic mouse model and analyzed immune conditions in the tumor microenvironment.

Since it is well known that there is an association between the level of tumor-infiltrating immune cells in the tumor microenvironment and patient prognosis, it is important to investigate the immunological situation in tumors. In this study to analyze the effects of anti-PD-1 mAb and 5-FU produced by APS001F/5-FC on immunological conditions in tumor microenvironment, we surveyed immunological cells in tumors. Especially, we directed our attention to cytotoxic T lymphocytes (CD8⁺) to potentially kill cancer cells, as well as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) that inhibit the antitumor immune response.

We hypothesized that 5-FU locally produced by APS001F in the tumor would affect the immune conditions of the tumor microenvironment, which, together with the direct antitumor activity of 5-FU, potentiates the antitumor effects of the combination therapy of APS001F/5-FC and anti-PD-1 mAb.

Materials and methods
Recombinant B. longum expressing cytosine deaminase (APS001F)

Frozen samples of APS001F, the recombinant B. longum strain expressing mutated cytosine deaminase with a mutation at the active site to increase the enzyme activity, were generated by Anaeropharma Science, Inc. (Tokyo, Japan). The host of APS001F is resistant to 5-FU as well as to spectinomycin, with a spectinomycin-resistant marker on the plasmid. APS001F was stored at −80 °C until use.

Anti-PD-1 mAb

Anti-PD-1 mAb (RMP1-14) was purchased from BioXcell.

Animals and cell lines

Animal experiments were executed in strict compliance with institutional guidelines and regulations. Research and technical procedures performed on animals under this study (#280055) were approved by the IACUC (Institutional Animal Care and Use Committee for Shinshu University). Female BALB/c mice were purchased from Japan SLC at 7 weeks of age and used for experiments at 8 weeks of age.

Mouse colon cancer cell line CT26 cells (CRL-2638™) were purchased from American Type Culture Collection. The cells were cultured using RPMI-1640 (4500 mg/L glucose) medium (FUJIFILM Wako Pure Chemical Corporation) containing 10% heat-inactivated fetal bovine serum (Equitech-Bio) and penicillin/streptomycin (Cosmo Bio) at 37 °C under 5% CO2.

Antitumor effects of APS001F/5-FC, anti-PD-1 mAb, and the combination in a CT26 model

CT26 cells (1 × 10⁵ cells) were subcutaneously (s.c.) inoculated in the right flank of mice. Mice were divided into groups with the average tumor volume of 100 mm³ on day 0. APS001F (1 × 10⁸ cfu) was i.v., 5-FC (Tokyo Chemical Industry) was intraperitoneally (i.p.), anti-PD-1 mAb (200 μg) was i.p., and maltose was i.p. administered according to the experimental schedule in the Figures 1a, 2a, 3a and 4a, respectively. Tumors were measured by mechanical calipers twice a week. Tumor volume (mm³) was calculated as length (mm) × width (mm) × height (mm)/2. Tumor growth inhibition (TGI, %) was calculated as (1 – mean tumor volume of test substance group/mean tumor volume of non-treated control group) × 100%. Mice with a tumor volume of >3000 mm³ were euthanized by cervical dislocation. For challenge trials, mice that had achieved complete tumor regression were inoculated s.c. with 2 or 5 × 10⁶ CT26 cells in the right flank to examine the engraftment of new tumors. All animal experiments were performed following approval by the Institutional Animal Care and Use Committee of Shinshu University School of Medicine (Matsumoto, Japan).
Figure 1. The antitumor effects of APS001F/5-FC, anti-PD-1 mAb, and their combination were evaluated in a syngeneic CT26 mouse model. (a) The experiment schedule. Mice were s.c. inoculated with $1 \times 10^5$ CT26 cells (day $-13$), and the stratification of mice was done (day 0). APS001F was administered i.v. at $1.0 \times 10^9$ cfu/mouse via the tail vein on day 3 with 200 mg maltose supplementation (days 3 through 16). 5-FC was administered i.p. at 250 mg/kg twice a day (500 mg/kg/day) on days 5 through 9 and days 12 through 16. Anti-PD-1 mAb was administered i.p. at 200 μg/mouse on days 1, 4, 8, and 11. Tumor volume was measured twice a week. (b) Change in tumor volume after treatment with APS001F/5-FC, anti-PD-1 mAb, and their combination. Results are the mean ± SEM of 9 mice. Analyses of 4 groups on day 21 were conducted using Tukey method of multiple comparisons. Means sharing a letter are not significantly different. TGI was calculated on day 21. (c) Survival rate of CT26-bearing mice after treatment with APS001F, anti-PD-1 mAb, and their combination. Mice with a tumor volume of $<3000$ mm$^3$ were defined as survivors, and a survival curve was prepared using the Kaplan–Meier method. *$P < .05$, **$P < .01$, ***$P < .001$.

Figure 2. The dose timing of APS001F/5-FC and anti-PD-1 mAb in combination therapy was investigated. (a) The experiment schedule of postdosing of APS001F/5-FC to anti-PD-1 mAb. Mice were s.c. inoculated with $1 \times 10^5$ CT26 cells (day $-13$), and the stratification of mice was done (day 0). APS001F was administered i.v. at $1.0 \times 10^9$ cfu/mouse via the tail vein on day 6 with 200 mg maltose supplementation (days 6 through 14). 5-FC was administered i.p. at 375 mg/kg twice a day (750 mg/kg/day) on days 8 through 14. Anti-PD-1 mAb was administered i.p. at 200 μg/mouse on days 1, 3, and 5. Tumor volume was measured twice a week. (b) The experiment schedule of predosing of APS001F/5-FC to anti-PD-1 mAb. Mice were s.c. inoculated with $1 \times 10^5$ CT26 cells (day $-13$), and the stratification of mice was done (day 0). APS001F was administered i.v. at $1.0 \times 10^9$ cfu/mouse via the tail vein on day 1 with 200 mg maltose supplementation (days 1 through 10). 5-FC was administered i.p. at 375 mg/kg twice a day (750 mg/kg/day) on days 3 through 9. Anti-PD-1 mAb was administered i.p. at 200 μg/mouse on days 4, 6, and 8. Tumor volume was measured twice a week. (c) Change in tumor volume after treatment with the combination. Results are the mean ± SEM of 8 mice. Analyses of 3 groups on day 21 were conducted using Tukey method of multiple comparisons. Means sharing a letter are not significantly different. TGI was calculated on day 21. (d) Survival rate of CT26-bearing mice. Mice with a tumor volume of $<3000$ mm$^3$ were defined as survivors, and a survival curve was prepared using the Kaplan–Meier method. **$P < .01$. 

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Figure 3. TIL analysis of CT26 cells in mice treated with APS001F/5-FC, anti-PD-1 mAb, and their combination. (a) The experiment schedule. Mice were s.c. inoculated with $1 \times 10^5$ CT26 cells (day -13), and the stratification of mice was done (day 0). APS001F was administered i.v. at $1.0 \times 10^9$ cfu/mouse via the tail vein on day 3 with 200 mg maltose supplementation (days 3 through 13). 5-FC was administered i.p. at 250 mg/kg twice a day (500 mg/kg/day) on days 5 through 9, and days 12 and 13. Anti-PD-1 mAb was administered i.p. at 200 μg/mouse on days 1, 5, 8, and 12. The mice were sacrificed and the tumors were excised on day 14. (b) Flow cytometric analysis of the tumor cells in mice engrafted with CT26 cells. Results are the mean ± SD of 8 mice. %CD45+ of total cells, %CD4+ of total cells, %Tregs (CD45+CD4+CD25+Foxp3+ cells) of CD4+ cells, %CD8+ of total cells, CD8/Treg ratio, %neutrophils (CD45+CD11b+Ly-6G+ cells) of total cells, and %TAMs (CD45+CD11b+Ly-6G-Ly-6Clow cells) of total cells were analyzed. Analyses of 4 groups were conducted using Tukey method of multiple comparisons. Means sharing a letter are not significantly different. (c) Histological analysis of tumor tissues in mice engrafted with CT26 cells. Blue arrowhead shows CD8+ cells (left panels) and Gr-1+ cells (right panels). Scale bars denote 0.1 mm. N, necrotic region; T, tumor proliferative region.

Flow cytometric analysis for immune cells in the tumor

Tumors were excised on day 14 according to the experimental schedule (Figure 3a) and dissociated using a gentleMACSTM Dissociator (Miltenyi Biotec). The number of cells in each tumor was counted with Scepter 2.0 software (Merck KGaA). Cell suspensions of each sample were aliquoted at $1 \times 10^6$ cells/tube for staining.

Cells were incubated with Zombie Violet Fixable Viability Dye solution (BioLegend) to identify live cells. T cells were stained by FITC antimouse CD4 antibody (RM4-5, BioLegend), APC antimouse CD8 antibody (53-6.7, BioLegend), PE/Cy7 antimouse CD45 antibody (30-F11, BioLegend), and APC/Cy7 antimouse CD25 antibody (PC61, BioLegend) along with anti-CD16/32 mAb (2.4G2, BioXCell or BD Biosciences) to reduce background FcγR binding. Intracellular molecules were stained by PE antimouse FoxP3 antibody (MF-14, BioLegend) after cell fixation and permeabilization. Myeloid cells were stained by FITC antimouse Ly-6G antibody (1A8, BioLegend), APC antimouse/human CD11b antibody (M1/70, BioLegend), and APC/Cy7 antimouse Ly-6C antibody (HK1.4, BioLegend).

Tumor cell surface molecules were stained by FITC antimouse H-2 antibody (M1/42, BioLegend), antimouse CD274 PE antibody (M1H5, eBioscience), APC antimouse I-A/E antibody (M5/114.15.2, BioLegend), PerCP/Cy5.5 antimouse CD80 antibody (16-10A1, BioLegend), and APC/Cy7 antimouse CD86 antibody (GL-1, BioLegend). All flow cytometric data were acquired by a BD FACS Canto II flow cytometer (Becton, Dickinson and Company) and analyzed using Kaluza software (Beckman Coulter). Gating strategy for immune cells and tumor cells in the grafted tumor is shown in Figure S1.

Histological analysis for CD8+ T cells and Gr-1+ cells in the tumor

Tumors excised on day 14 were embedded in O.C.T. compound (Sakura Finetek), made into frozen blocks in liquid nitrogen, and stored at -80 °C. Frozen sections were prepared from the frozen blocks using a cryo-microtome, subjected to their respective stains, and examined microscopically. The slides were stained with Biotin anti-mouse Ly-6G/Ly-6C (Gr-1) (RB6-8C5, BioLegend) and APC/Cy7 antimouse CD8a antibody (GL-1, BioLegend). All flow cytometric data were acquired by a BD FACS Canto II flow cytometer (Becton, Dickinson and Company) and analyzed using Kaluza software (Beckman Coulter). Gating strategy for immune cells and tumor cells in the grafted tumor is shown in Figure S1.
ABC Kit (Vector Laboratories) and Peroxidase Stain DAB Kit (Nacalai Tesque). After the DAB reaction, the slides were stained by Mayer's hematoxylin solution (Fujifilm Wako Pure Chemical Corporation) for counter-staining of cell nuclei. The image from slides was acquired using microscope (Leica Microsystems).

Statistical analysis
Data from the tumor volume and flow cytometric analyses were evaluated using Tukey's multiple comparison test at a significance level of $P < 0.05$. Means sharing a letter (for example, a–a, b–b) are not significantly different. In survival tests, mice with a tumor volume of $>3000$ mm$^3$ were defined as survivors, and survival curves were prepared using the Kaplan-Meier method. Significance was tested using the log-rank test at a level of $P < 0.05$. All data was analyzed using GraphPad prism 6 (GraphPad Software).

Results
Antitumor effects of the monotherapy of APS001F/5-FC or anti-PD-1 mAb, and their combination
The antitumor effects of APS001F/5-FC alone, anti-PD-1 mAb alone and their combination were evaluated in BALB/c mice engrafted s.c. with CT26 cells according to the experimental protocol depicted in Figure 1a. We used clone RPMI-14 of anti-PD-1 mAb, since this clone is reportedly effective in a mouse tumor model (Grasselly et al. 2018; Markowitz et al. 2018).

On day 21 following grouping on day 0, APS001F/5-FC demonstrated a significant inhibition of tumor growth (TGI: 55.1%), and anti-PD-1 mAb showed a modest inhibition (TGI: 23.8%) over the nontreated control. The combination treatment, where anti-PD-1 mAb was i.p. dosed before APS001F, exhibited a significant inhibition of tumor growth (TGI: 84.5%) over the nontreated control treatment (Figure 1b).

APS001F/5-FC significantly prolonged survival versus the nontreated control. The combination of APS001F and anti-PD-1 mAb achieved significantly longer survival in comparison with the nontreated control and both monotherapies (Figure 1c).

Complete tumor regression was observed in 1 of the 9 animals in the combination group. To assess memory response, that animal was rechallenged with CT26 cells (5 times the number of initially inoculated cells) in the same area as the initial inoculation on day 44. No tumor growth was observed up to day 84 (data not shown).

Comparing antitumor effects between pre- and post-APS001F to anti-PD-1 mAb
Timing of APS001F, followed by 5-FC i.p. administration, and anti-PD-1 mAb administration in the combination therapy was investigated in the CT26 mouse model according to the dose schedule described in Figure 2a and b. In combination therapy of cancer, the timing of dosing drugs is usually examined, so that we followed such convention. Since the combination of APS001F/5-FC and anti-PD-1 mAb exhibited superior antitumor effect to each monotherapy (Figure 1), and we increased 5-FC dose to 750mg/kg that corresponds to the dose in clinical trial. Both of APS001F before and after anti-PD-1 mAb significantly inhibited the tumor growth (TGI in APS001F/5-FC before anti-PD-1 mAb: 68.8%, TGI in APS001F/5-FC after anti-PD-1 mAB: 64.1%), and there was no difference between the 2 groups (Figure 2c). Survival rate was significantly increased in both groups over the nontreated control (Figure 2d). Complete tumor regression was observed in 1 mouse in the experiment (Figure 2d). Rechallenge with tumor cells for the mouse also failed to form tumor as observed when the experiment of Figure 1 was done (data not shown).

Analysis of tumor-infiltrating leukocytes (TIL) and distribution of CD8$^+$ T cells and Gr-1$^+$ cells in the tumor
Since antitumor effects were observed for all combination dosing of APS001F/5-FC and anti-PD-1 mAb, TIL analysis was performed following the administration of APS001F/5-FC on day 3 and anti-PD-1 mAb on days 1, 5, 8, and 12 (Figure 3a). Tumor tissues were excised on day 14 and subjected to flow cytometric analysis and immunohistochemical staining.

The combination therapy significantly increased the percentage of CD45$^+$ cells among total cells to as high as 80%, when compared with the nontreated control and both monotherapies. In some animals of the combination group, the tumor tissues were occupied by CD45$^+$ cells (Figures 3b and S2A). CD4$^+$ T cells among total cells significantly increased in groups of anti-PD-1 mAb and the combination over the nontreated control. Of note was that regulatory T cells (Tregs) among CD4$^+$ T cells significantly decreased only in the combination (Figures 3b and S2A). CD8$^+$ T cells did not differ in all treatment groups (Figures 3b and S2A). As a result, CD8/Treg was increased (Figures 3b and S2A). Immunohistochemistry analysis revealed that CD8$^+$ T cells were present in the proliferative regions of tumor in the combination (Figure 3c).

The ratio of CD11b$^+$Ly-6G$^+$ cells to total cells significantly increased in APS001F/5-FC and the combination (Figures 3b and S2A). These cells were observed to localize at the boundary between necrotic and proliferative regions (Figure 3c). The ratio of TAMs (CD11b$^+$Ly-6G$^-$Ly-6C$^{int}$) to total cells did not significantly differ from that of the nontreated control (Figures 3b and S2A).

Flow cytometric analysis for tumor cell surface molecules following the combination dosing
In the analysis on day 14 as illustrated in Figure 3a, the expression level of H-2 (MHC class I) was significantly increased in APS001F/5-FC, while that of the combination did not (Figure 4). The expression of I-A/I-E (MHC class II) did not differ among all groups. The expression level of CD80 was significantly higher in APS001F/5-FC and the combination than in anti-PD-1 mAb. CD86 expression level increased significantly in all treatment groups over the nontreated control. The expression level of PD-L1 rose significantly in the combination but not in the other groups (Figures 4 and S2B).

Discussion
In the present study, monotherapy with APS001F/5-FC significantly inhibited the tumor growth in a CT26 tumor model. The antitumor activity was potentiated by the combination of APS001F/5-FC with anti-PD-1 mAb. The combination treatment not only suppressed the tumor growth (Figures 1b and 2c) but also significantly prolonged survival of tumor-bearing mice (Figures 1c and 2d). Both pre- and postdosing of APS001F, followed by i.p. 5-FC administration, with anti-PD-1 mAb significantly suppressed the tumor growth (Figure 2c), suggesting that the combination effect is not dose-schedule dependent.
survival was observed for mice receiving the combination treatment (Figures 1c and 2d). Tumors were completely regressed in these mice. Since the rechallenge of CT26 cells for these mice failed to form tumor, it is likely that memory T cells specific for CT26 cells are present in mice dosed with the combination therapy. The results indicate that APS001F/5-FC has an immunomodulatory effect in addition to a direct antitumor effect.

The recombinant B. longum to overexpress cytosine deaminase gene localized and produced a substantial amount of 5-FU only in the tumor region of the rat model (SasakI et al. 2006). APS001F is an analog to this recombinant B. longum and produced 5-FU in the tumor region of the mouse model (data not shown). Recent studies suggest that chemotherapeutic agents, such as 5-FU, play an immunomodulatory role by killing MDSC in addition to a direct cytotoxic activity (Vincent et al. 2010; Zitvogel et al. 2013), which is also associated with side effects as represented by myelosuppression (Kadoyama et al. 2012). Repeated cycles of 5-FU inhibited the growth of CT26 tumor without improvements of survival rate due to impaired antitumor immunological functions, compared with a single cycle of dosing (Wu et al. 2016). Thus, as for tumor immunology, systemically administered 5-FU has not only pros to remove MDSC but also cons to induce myelosuppression, whereas APS001F/5-FC system could improve the situation because it produces 5-FU only in the tumor.

It is hypothesized that the potentiated antitumor effects by the combination therapy are likely attributable to induce advantageous antitumoral immunological conditions, as described below, to allow anti-PD-1 mAb to exert potentiated antitumor effects, together with the direct antitumor activity of 5-FU. Firstly, CD45+ cells among total cells increased in APS001F/5-FC and the combination, and CD4+ cells among total cells increased in anti-PD-1 mAb and the combination, respectively (Figures 3b and S2A). The increased number of CD45+ cells in both APS001F/5-FC and the combination indicate no occurrence of myelosuppression. No myelosuppression has been consistently observed in multiple preclinical experiments using APS001F/5-FC (data not shown). Second, CD8+ cells did not change in all groups, while Tregs among CD4+ cells dramatically decreased only in the combination group, irrespective of no change in the other groups. Thus, the increased CD8/Treg ratio could lead to enhanced antitumor effects (Selby et al. 2016; Mahne et al. 2017) of anti-PD-1 mAb used in the combination. The increase in CD8/Treg in the combination is very interesting phenomenon but the mechanism remains to be elucidated.

In our study, CD11b+Ly-6G+ cells among total cells increased in APS001F/5-FC, and further increased in the combination over APS001F/5-FC (Figures 3b and S2A). We presumed that CD11b+Ly-6G+ cells were mainly neutrophils rather than MDSC by considering the facts (1) that increase of MDSCs in the tumor usually correlates with tumor progression (Meyer et al. 2011; Ostrand-Rosenberg and Fenselau 2018; Groth et al. 2019), (2) that MDSC has been reported to be killed by 5-FU (Vincent et al. 2010), (3) that according to our experience, morphologically apparent neutrophils usually gather in the vicinity of APS001F, and (4) that in this experiment, the localization of Gr-1+ cells in the tumor was observed at the necrotic site or at the border between the necrotic site and the tumor (Figure 3c) where Bifidobacterium is usually detected (SasakI et al. 2006). The neutrophils may contribute to antitumor effects, although conflicting data are described that neutrophils play an antitumor role as well as a tumor promoting role (Schmidt et al. 2005; Friidlenber et al. 2009; Grecian et al. 2018; Masucci et al. 2019).

The combination therapy significantly increased PD-L1 expression over the monotherapies and the nontreated control (Figure 4b). CD80 and CD86 are expressed on tumor cells to escape from immune attack (Tirapu et al. 2006; Sun et al. 2012). In the present study, APS001F/5-FC and the combination treatment increased the expression of CD80 and CD86 compared with anti-PD-1 mAb and the nontreated control (Figure 4b). Both immune costimulator CD28 and immune suppressor CTLA-4 bind CD80 and CD86, while CTLA-4 binds to CD80 and CD86 with much higher affinity than CD28, consequently leading to downregulate the immune-stimulating function of CD28 (Seidel et al. 2018).
Combination of CD80 or CD86/CTLA-4 blockade like anti-CTLA-4 mAb with APS001F/5-FC may lead more antitumor efficacy.

In conclusion, our findings are promisingly indicating the design of the combination therapy of APS001F/5FC and anti-PD-1 mAb.

Supplementary material
Supplementary material is available at Bioscience, Biotechnology, and Biochemistry online.

Author contribution
K.S. and S.T. designed the experiments. K.S., T.M., Y.S., and H.S. performed experiments. K.S., T.M., and T.N. analyzed data. K.S. drafted the manuscript. T.N. and S.T. revised the manuscript. All authors have read and approved the final manuscript.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Disclosure statement
S. Taniguchi is a Science Advisor of Anaeropharma Science, Inc.

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