Original Article

Soy isoflavone genistein attenuates the efficacy of immune checkpoint therapy in C57BL/6 mice inoculated with B16F1 melanoma and a high PD-L1 expression level reflects tumor resistance

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Immune checkpoint therapy has been shown to be an effective therapy for many types of tumors. Much attention has been paid to the development of an effector target would be helpful for immune checkpoint therapy. Genistein has been shown to have an anti-tumor effect both *in vitro* and *in vivo*. In this study, we examined the effect of genistein on immune checkpoint blockade therapy against B16F1 melanoma tumors. Mice treated with genistein or anti-programmed death (PD)-1 antibody showed a significant decrease in tumor growth. However, treatment with genistein had no effect on or attenuated the efficacy of immune checkpoint therapy. The percentages of T cell receptor (TCR)β$^+$CD4$^+$ and TCRβ$^+$CD8$^+$ cells and the concentrations of interferon-γ and tumor necrosis factor-α in tumor tissue were not different among the experimental groups. A significant difference was also not found in microbe composition. Interestingly, a high expression level of PD-ligand (L)1 closely reflected the outcome of therapy by genistein or anti-PD-1 antibody. The study showed that a combination of genistein treatment does not improve the effect of immune blockade therapy. It also showed that a high PD-L1 expression level in tumors is a good prediction maker for the outcome of tumor therapy.

***Key Words*: genistein, tumor, immune checkpoint therapy, PD-1, PD-L1**

The first reported case of immunotherapy against cancer was inoculation of erysipelas toxin into sarcoma in 1891.[1] Many types of cancer immunotherapy have been developed in the past century. BCG, cytokine therapy, tumor antigen (Ag)-based vaccination, and transplantation of activated autoimmune cells have been developed and conducted in a clinical trial.[2] However, satisfactory results have not been obtained. Immune therapy for cancer was dramatically changed by the finding that binding of programmed death (PD)-1 to PD-ligand (L)1 molecule suppresses T cell activation.[3,4] Immunotherapy by immune checkpoint inhibitors such as anti-PD-1, PD-L1 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) monoclonal antibodies (mAbs) has emerged as a promising treatment for cancer patients in recent years.[5] However, the current checkpoint blockade therapy has limited success in certain types of cancers with no more than 40% success rates overall.[6]

Soy intake has been shown to prevent hormone-related cancers such as breast cancer and prostate cancer in humans.[7,8] Soy isoflavones are candidates for the preventive components. It has been shown that the soy isoflavone genistein inhibits *in vitro* proliferation of tumor cells.[9] Treatment with genistein in mice that had been inoculated with B16F1 melanoma enhanced NK and cytotoxic cell activity and then suppressed tumor growth.[10]

Much attention has be paid to augmentation of the effect of immune checkpoint therapy. Many approaches have been tried including combinations of chemotherapy, cytokines, inhibitors, and radiotherapy.[11–15] It has been shown that food-derived components are a useful agent in human health and safety rather than medical drugs. Natural compounds have low toxicity and exert anti-tumor and immunomodulatory effects. The combination of immune checkpoint inhibitors with natural products may provide a novel strategy for treatment of tumors. In this study, we investigated the effect of the soy isoflavone genistein on immune checkpoint therapy and explored the underlying mechanism.

Materials and Methods

Mice and diets. Six-week-old female C57BL/6 mice (Japan SLC, Shizuoka, Japan) were maintained under specific pathogen-free conditions with a 12-h light:dark cycle at 25 ± 2°C and 55 ± 10% relative humidity. The mice were given free access to water and food throughout the experiment. The mice were maintained on a control diet (No. D10012G; Research Diets Inc., New Brunswick, NJ). All studies were performed in accordance with the ethical guidelines for animal experimentation by the Institute of Biomedical Sciences, Tokushima University, Japan and were approved by the institution review board of the animal ethics committee.

Treatment with genistein and/or anti-PD-1 monoclonal antibody (mAb). Genistein was obtained from Tokyo Chemical Ltd (Tokyo, Japan). A hybridoma, RMP1-14, producing anti-PD-1 mAb was injected into BALA/c *nu/nu* mice. The anti-PD-1 mAb was purified from ascites fluids by the caplic acid method.[16] Mice were treated with genistein at a dose 50 mg/kg weight by gavage from 7 days before to the end of the experiment. Administration of anti-PD-1 mAb (0.5 mg/mouse) was done on days 17, 20, and 23.

Flow cytometric analysis. Tumor tissue was chopped and incubated for 60 min and then shaken continuously at 37°C in a dissociation cocktail: 0.1% collagenase (Wako, Osaka, Japan)

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and 10 U/ml DNase (Wako) in serum-free medium. The dissociated cell suspension was centrifuged on a discontinuous 44–70% Percoll gradient. Cells in the interphase were collected. The cells were stained with fluorescein isothiocyanate-conjugated anti-CD8 mAb, phycoerythrin-conjugated anti-CD4 mAb, peridinin-chlorophyll-protein-conjugated anti-CD45 mAb and allophycocyanin-conjugated anti-T cell receptor (TCR)β mAb. All of the Abs were purchased from eBioscience. Flow cytometric analysis was performed on Guava easyCyte using Guava Incyte software (Merck Millipore, Darmstadt, Germany).

Cytokine measurement. Tumor tissue was homogenated with phosphate-buffered saline and debris was removed by centrifuging at 10,000 *g*. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α in the supernatants were quantified using a mouse IFN-γ (eBioscience, SanDiego, CA) and TNF-α (eBioscience) enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Microbiota composition. Fresh fecal samples were collected from the cecum at the end of the experiment. DNA was extracted using a NucleoSpin DNA Stool (MACHEREY-NAGEL, GmbH Co., KG, Germany). Isolated DNA was analyzed using 16S rRNA sequences to investigate the microbial composition. The 16S RNA genes were amplified by PCR using composite-specific bacterial primers for the V3–V4 region and sequenced by a next-generation sequencer (Genome Lead Co., Takamatsu, Japan)

Western blot analysis. Tumor tissue was lysed with lysate buffer. Proteins were loaded on SDS-PAGE and then transferred onto polyvinylidene fluoride membranes. The membranes blocked with 5% nonfat milk were probed with primary Abs at 4°C overnight and then incubated with a horseradish peroxidase-conjugated secondary Ab. Rabbit anti-PD-L1 (cat. #2177812) and mouse anti-β actin (cat. #66009) were purchased from Bioss (Woburn, MA) and Proteintech (Chicago, IL), respectively.

Statistics. Data are shown as means ± SD. The results were analyzed by the *t* test between control and experimental groups. When the *p* value was less than 0.05, we defined the difference as significant.

Results

Treatment with genistein or anti-PD-1 mAb suppresses the growth of B16F1 tumors, but their combination does not improve their effects. C57BL/6 mice were inoculated with B16F1 melanoma cells and treated with genistein and/or anti-PD1 mAb. Mice that were treated with genistein or anti-PD-1 mAb showed a reduced tumor volume and significant differences in tumor volume were observed at days 21 and 24 compared to those in the control group. A combination of genistein and anti-PD-1 mAb treatment did not suppress tumor growth more than a single treatment. Notably, a significant reduction in tumor volume was observed at day 21 in mice with both genistein and anti-PD-1 mAb treatment, but the significant difference was lost at day 24 by the combination treatment (Fig. 1 A and B).

Immune cell characterizations in mice treated with genistein and/or anti-PD-1 mAb. We first investigated the T cell subsets that had infiltrated into the tumor to explore the protective mechanism for tumor growth. The percentage of $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ cells was not different between the control and treated groups (Fig. 2). Although we further determined the effector cytokines, IFN-γ and TNF-α, a significant difference in the concentrations of these cytokines was not observed (Fig. 3).

Analysis of microbiota in mice treated with genistein and/or anti-PD-1 mAb. Recent evidence suggests that microbiota play an important role in the efficiency of immune checkpoint therapy. We analyzed the composition of microbiota at the phylum level in mice treated with genistein and/or anti-PD-1 mAb. As shown in Fig. 4A, a significant difference was not observed in the composition of microbiota between the 4 groups. In addition to the analysis of phylum level composition, we determined alfa and beta diversity in microbiota. Although alfa diversity was not different among the 4 groups (Fig. 4B, $p = 0.447$), beta diversity in the microbiota of the experimental groups seems to be different (Supplemental Fig. 1A*, $p<0.07$).

Expression of PD-L1 in the tumor is correlated with tumor resistance. PD-L1 in the tumor drives an inhibitory signal to T cells via PD-1 molecules. We focused on the expression of PD-L1 molecules and determined their expression levels. The expression levels of PD-L1 in mice treated with genistein and mice treated with anti-PD-1 mAb were significantly higher than the expression level in control mice. Mice that were treated with both genistein and anti-PD-1 mAb showed reduced PD-L1 expression compared to that in mice treated with genistein or anti-PD-1 mAb alone. The results suggest that the expression of PD-L1 in tumors reflects the growth status of the tumor and the outcome of the therapy.

Discussion

Immune checkpoint therapy against cancer is a novel approach and has been approved by the FDA. However, current checkpoint blockade therapy has limited success in certain types of cancers. Many approaches for improving therapy outcomes by immune checkpoint blockage such as the use of chemotherapy, radiotherapy, and tyrosine kinase inhibitors have been attempted.[11–15] In this study, we chose the food-derived compound genistein and examined the effect of genistein on immune checkpoint therapy. We found that treatment with genistein or anti-PD-1 mAb suppresses the growth of B16F1 melanoma but that a combination of these two treatments cannot improve the protective activity (Fig. 1). It has been shown that inhibition of tumor growth by immune checkpoint therapy is correlated with activation of $CD4^+$ and $CD8^+$ T cells.[17,18] To elucidate the mechanism, levels of infiltrated lymphocyte subset and cytotoxic cytokines were determined. Unexpectedly, percentages of $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ cells and concentrations of IFN-γ and TNF-α were not different between the control and treatment groups (Fig. 2 and 3).

Recent studies have highlighted key roles of gut microbiota in mediating tumor responses to chemotherapeutic agents and in immunotherapies targeting PD-L1 or CTLA-4.[19–21] Since we could not find a relationship between tumor resistance and T-cell activation, we next analyzed microbiota compositions in the four groups. A significant difference in beta-diversity was not observed but it tended to show different patterns in the four groups (Supplemental Fig. 1A*). Although we compared microbiota compositions at the phylum, class, order, family and genus levels (Fig. 4A and Supplemental Fig. 1B and C*), no difference was found. In a mouse model, *Bifidobacterium fragilis* and *Akkermansia muciniphila* have been shown to improve the efficacy of checkpoint blockade immunotherapy.[20–22] We could not find a difference in these bacteria among the experimental groups (data not shown).

It has been shown that several food-derived components enhance therapeutic efficacy through immune checkpoint treatment. Leteolin and its derivative apigenin improve anti-tumor immunity in KRAS-mutant lung cancer.[23] The mechanism of improvement of anti-tumor immunity is thought to be down-regulation of PD-L1 expression in the tumor. In another study, caffeine was shown to be effective in anit-PD-1 mAb therapy in B16F10 melanoma-inoculated mice.[17] In those two studies, inhibition of tumor growth was associated with an increment of T cell functions. Independent of immune check point therapy, the dietary phytochemicals apigenin and omega-3 exert anti-tumor effects and the actions of their effects are mediated by a decre-

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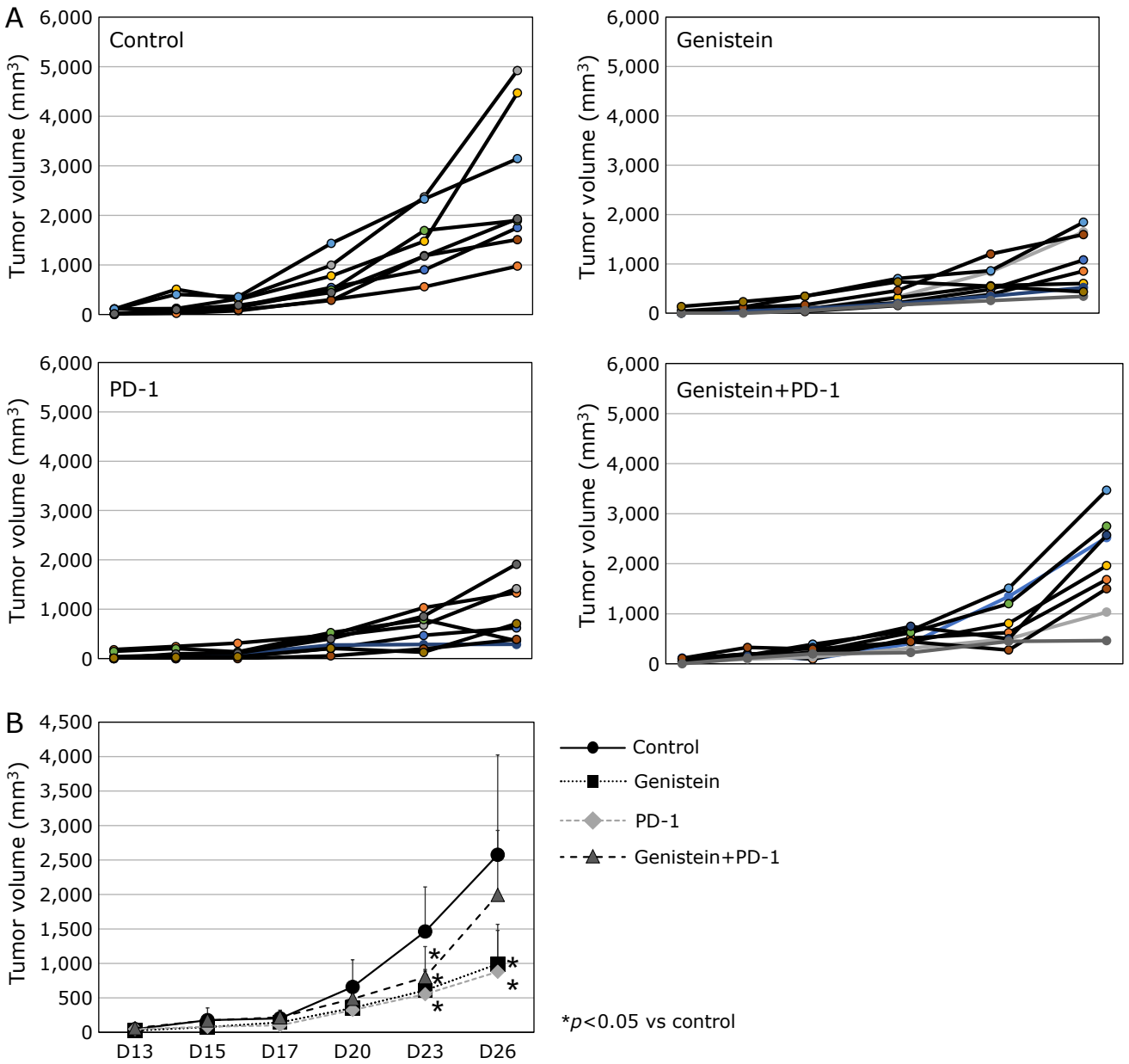

Fig. 1. Treatment with genistein or anti-PD-1 mAb suppresses the growth of B16F1 tumors, but the combination of genistein and anti-PD-1 mAb does not improve the effects. C57BL/6 mice were treated with 50 mg/kg body weight of genistein 7 days before inoculation with 10^5 B16F1 melanoma cells. Anti-PD-1 mAb was administered at day 17, 20, and 23 after tumor inoculation. The volume of tumors each mouse from day 13 to day 26 are shown (A). The tumor volumes in the control, genistein, anti-PD-1 mAb and genistein + anti-PD-1 mAb groups from day 13 to day 26 are shown as means ± SD (B). A statistical difference was analyzed between the control and treated groups. *p<0.05. **p<0.01.

ment of PD-L1 expression.[24–26] In the case of omega-3, it has been shown that treatment of tumor cells with omega-3 induced ubiquitination of PD-L1 molecules and then degraded them.[26] Independent of PD-L1 mechanism, lipid-soluble polyphenols from potato has been shown to suppress tumor growth *in vitro* and enhance chemosensitivity *in vivo*.[27]

Human clinical studies have shown that expression of PD-L1 in the tumor is a promising biomarker for prognosis of patients with breast cancer, non-small-cell cancer and gastric cancer.[28–30] The expression level of PD-L1 is associated with overall survival. Although we did not find differences in a T cell subset, cytokine production and microbiota between the control and treatment groups, we found a significant association in the expression of PD-L1 (Fig. 5). However, it is not clear whether the regulation of PD-L1 is a direct effect of genistein and/or anti-PD-1 mAb treatment or an indirect effect. There have been interesting results of studies showing that the expression of PD-L1 in tumor-infiltrating lymphocytes is related to better survival of patients with cancer.[31,32] We cannot distinguish the expression of PD-L1 among tumors and tumor-infiltrating lymphocytes. Further studies were needed to explore this point, and elucidation of the mechanism by which a high expression level of PD-L1 confers anti-tumor immunity could provide a new insight for immune check blockade.

We did not observe an additional effect of genistein on the outcome of immune checkpoint therapy (Fig. 1). Equol is one of soy isoflavones and is structurally similar to genistein. Equol does not show anti-tumor action but exerts anti-tumor action

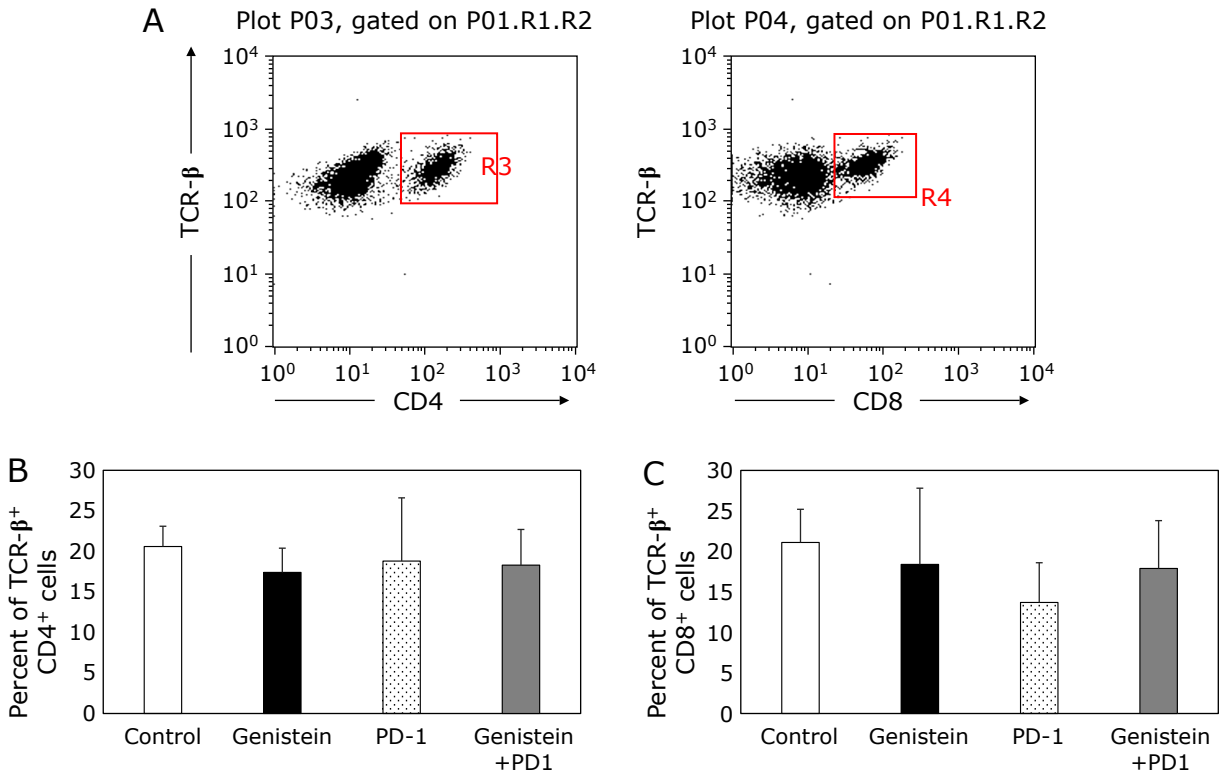

Fig. 2. T cell subsets infiltrating B16F1 tumors in mice treated with genistein and/or anti-PD-1 mAb. Lymphocytes were prepared from the tumor as described in Materials and Methods. Lymphocytes were stained with FITC-conjugated anti-CD8 mAb, PE-conjugated anti-CD4 mAb, PerCP-conjugated anti-CD45 mAb, and APC-conjugated anti-TCRβ mAb. Stained cells were analyzed by gating on $CD45^+$ cells by flow cytometric analysis. Percentages of $TCR\beta^+CD4^+$ (A) and $TCR\beta^+CD8^+$ (B) cells were determined by flow cytometry. Results are shown as means ± SD.

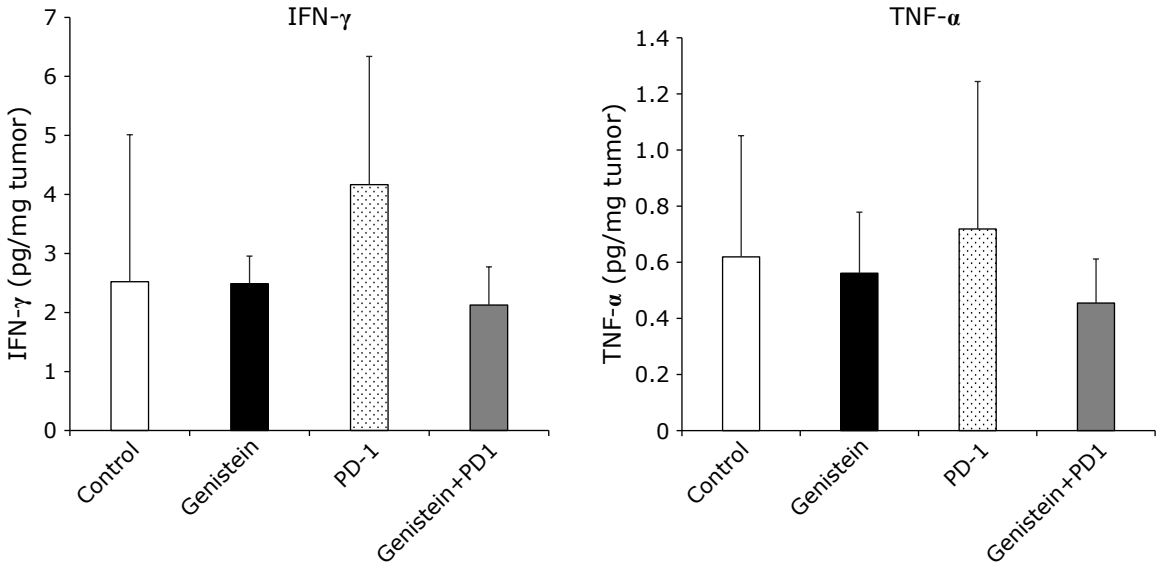

Fig. 3. Concentrations of IFN-γ and TNF-α in tumors. Tumor tissue was homogenated and centrifuged to remove debris. The concentrations of IFN-γ and TNF-α in the supernatant were determined by ELISA methods. Results are shown as means ± SD.

when used in combination with anti-PD1 mAb treatment.[33] In this mechanism, equol might enhance T cell receptor activation in $CD8^+$ T cells via estrogen receptor (ER)β. ERs α and β, which are encoded by different genes, mediate the diverse physiological effects of estrogens. Soy isoflavones have been shown to preferentially bind to the ERβ rather than tothe ERα.[34] The differential effects of genistein and equol on immune checkpoint therapy is not clear. Although the binding affinity of genistein and that of equol to ERβ is the same, transcription ability as assessed by the proliferation response in ER-positive MCF-7 cells by equol is

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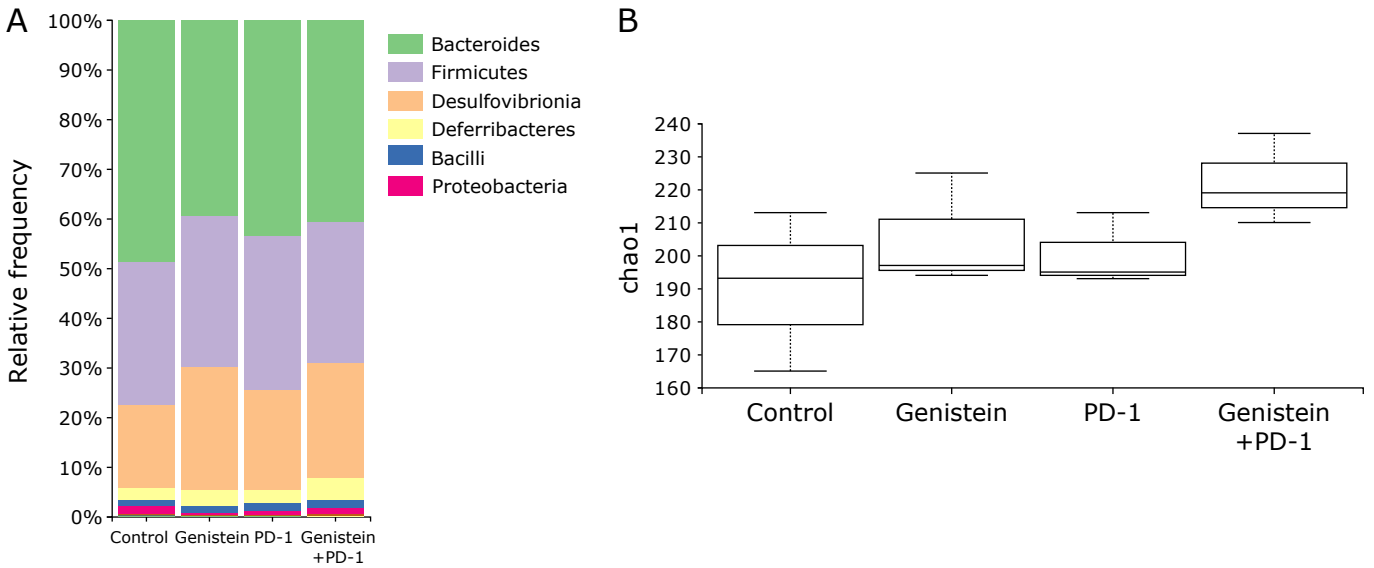

Fig. 4. Analysis of microbiota in mice treated with genistein and/or anti-PD-1 mAb. The compositions of microbiota in mice treated with genistein and/or anti-PD-1 mAb were compared at the phyla levels (A). The composition of the microbiota was analyzed by beta diversity (B). See color figure in the on-line version.

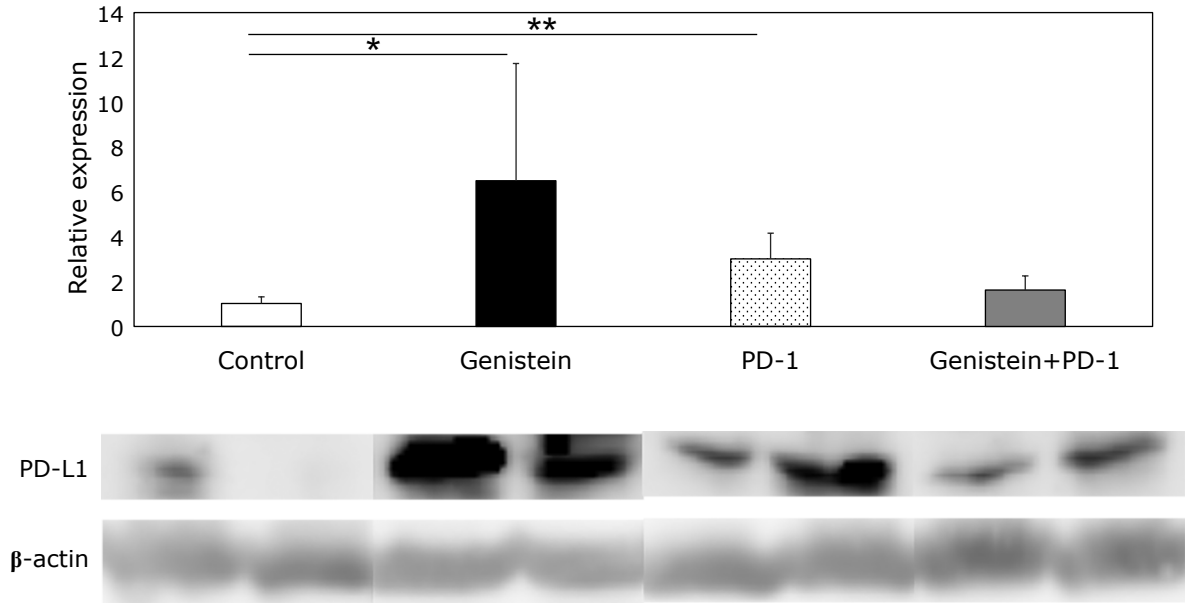

Fig. 5. High expression level of PD-L1 is a novel marker for prediction of the outcome of treatment. Expression of PD-L1 in tumors from mice treated with genistein and/or anti-PD-1 mAb was determined by Western blot analysis. The expression level of PD-L1 was analyzed by the expression of β-actin as an internal control. Results are shown as means ± SD. *p<0.05. **p<0.01.

stronger than that by genistein.[35]

An attenuation of immune blockade therapy has been reported in isothiocyanates, which are found in cruciferous vegetables.[36] Combination treatment of isothiocyanates and anti-PD-1 mAb weakened the sensitivity of tumor cells to anti-PD-1 therapy. The mechanism for reducing the effect of immune blockade inhibitors is mediated by TP63 inducing up-regulation of PD-L1 in tumors. Although treatment with genistein partially abolished the effect of immune blockade inhibitors (Fig. 1), up-regulation of PD-L1 was not observed (Fig. 5). Therefore, the suppressive effects of genistein and isothiocyanates on immune blockade inhibitors might be different.

In conclusion, the soy isoflavone genistein does not improve the efficacy of immune checkpoint blockage. We found that a high expression level of PD-L1 in tumors is a useful clinical prognostic maker for cancer therapy.

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Abbreviations

Ab	antibody
CTLA-4	cytotoxic T lymphocyte-associated protein 4
ELISA	enzyme-linked immunosorbent assay
L	ligand
IFN	interferon
m	monoclonal
PD	programmed death
TCR	T cell receptor
TNF	tumor necrosis factor

Conflict of Interest

No potential conflicts of interest were disclosed.

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