Integrative Cancer Therapies
Volume 19: 1–13

DOI: 10.1177/1534735419900798
journals.sagepub.com/home/ict
SAGE

Japanese Kampo Medicine Juzentaihoto Enhances Antitumor Immunity in CD1d$^{-/-}$ Mice Lacking NKT Cells

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Abstract

Although the Japanese traditional herbal medicine (Kampo), Juzentaihoto (JTT), has been reported to have antitumor effects in several tumor models, its role in tumor immunology remains controversial. In the present study, we tested whether oral administration of JTT enhances antitumor immunity in CD1d$^{-/-}$ mice, in which immunosuppression was partially relieved due to the lack of NKT cells. In a subcutaneous murine syngeneic CT26 colorectal tumor model, JTT had no impact on tumor growth in wild type (WT) BALB/c mice. However, the growth rate of tumors was significantly slower in CD1d$^{-/-}$ mice than in WT mice. Surprisingly, JTT significantly delayed tumor growth in such CD1d$^{-/-}$ mice. In vivo depletion of CD8^{+} T cells revealed that CD8^{+} T cells are required for JTT's antitumor activity. Moreover, tumor-reactive cytotoxic T-lymphocytes were detected exclusively in JTT-treated mice with well-controlled tumors. JTT did not affect the number of tumor-infiltrating CD4^{+} regulatory T cells. On the contrary, JTT increased the degranulation marker CD107a^{+} CD8^{+} T cells and decreased Ly6G^{+} Ly6C^{lo} polymorphonuclear myeloid-derived suppressor cells in tumor-infiltrating lymphocytes, most probably contributing to the suppression of tumor growth in JTT-treated mice. Nonetheless, JTT had no impact on the proportion of monocytic myeloid-derived suppressor cells. In conclusion, our results indicate that in the absence of NKT cells, JTT augments antitumor immunity by CD8^{+} T cells, suggesting that this Kampo medicine is a promising anticancer adjuvant when negative immune regulation is partially relieved.

Keywords

Juzentaihoto, CD1d$^{-/-}$ mice, CD8^{+} T cells, NKT cells, regulatory T cells, myeloid-derived suppressor cells

Submitted May 4, 2019; revised November 30, 2019; accepted December 21, 2019

Introduction

Juzentaihoto (JTT; Shi-Quan-Da-Bu-Tang in Chinese and Sipjeondaebo-Tang in Korean) is a traditional Japanese herbal medicine, also known as *Kampo*, comprising 10 herbs (Table 1). In Japan, it has been traditionally used as an additional therapy in patients with anemia, fatigue, anorexia, scaly skin, and dry mouth.[1] JTT has also been frequently prescribed to cancer patients to mitigate side effects caused by current standard cancer treatments, including surgery, chemotherapy, and radiotherapy.[2-4] Furthermore, several studies performed in mouse tumor models have shown that JTT can prevent cancer occurrence[5,6] and suppress liver[7] and lung metastases.[8,9] In patients with hepatocellular carcinoma, it has been shown that JTT can extend the length of intrahepatic recurrence-free survival after surgical treatment.[10] However, some earlier murine studies have shown that JTT alone did not always have antitumor effects.[11,12] Moreover, in advanced pancreatic cancer patients, JTT failed to augment antigen-specific antitumor immunity as a personalized peptide vaccine.[13] Therefore, the antitumor effects of JTT remain controversial, and its precise mechanisms are yet to be elucidated.

Recent progress in cancer immunology has shown that negative immune regulation is a critical barrier to combating cancers. Tumor cells are capable of developing numerous

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Table 1. Composition of Juzentaihoto.

Crude Drug	Botanical Origin	Ratio (g)
Angelicae radix	Root of *Angelica acutiloba* Kitagawa	3.0
Poria	Fungus of *Poria cocos* wolf	3.0
Rehmanniae radix	Root of *Rehmannia glutinosa* Libosch var purpurea Makino	3.0
Ginseng radix	Root of *Panax ginseng* CA Meyer	3.0
Cinnamomi cortex	Bark of *Cinnamomum cassia* Blume	3.0
Paeoniae radix	Root of *Paeonia lactiflora* Pallas	3.0
Astragali radix	Root of *Astragalus membranaceus* Bunge	3.0
Glycyrrhizae radix	Root of *Glycyrrhiza uralensis* Fisher and DC	1.5
Cnidii rhizome	Rhizome of *Cnidium officinale* Makino	3.0
Atractylodis lanceae rhizome	Rhizome of *Atractylodes lancea* DC	3.0

mechanisms to evade the immune system and progress. Among them, $CD4^+$ $CD25^+$ regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSC), and type II NKT cells represent major cell types responsible for immunosuppression in tumor-bearing hosts.[14-16] Treg cells can suppress the effects of anticancer immunotherapy, and there is a correlation between their presence at local tumor sites and an unfavorable prognosis.[17,18] In cancer-bearing hosts, the number of MDSC increases and can inhibit T cell responses.[19,20] NKT cells comprise 2 subsets: type I NKT cells promote antitumor immunity, and type II NKT cells suppress tumor immunity. The simultaneous stimulation of both NKT cells in wild type (WT) immune-competent mice diminished protection against tumors in vivo in 2 different tumor models,[21] suggesting that type II NKT cells dominate type I NKT cells. In contrast, in a study using $J\alpha\text{-}18^{-/-}$ mice lacking type I NKT cells, but still retaining type II NKT cells, the absence of type I NKT cells resulted in an increase in the suppression by type II NKT cells,[21] suggesting that type I NKT cells somewhat reduce the suppressive effect of type II NKT cells. Thus, the 2 subsets cross-regulate each other, forming an immunoregulatory axis.[21] Although the blockade of such immunosuppressors is a promising strategy for enhancing antitumor immunity, targeting one cell type is not always sufficient for tumor rejection because of the redundancy of immune regulatory cells. In a subcutaneous murine syngeneic CT26 colorectal tumor model, Treg cells are known to be key immunosuppressors; their depletion completely abrogated tumors in WT mice.[22-24] However, in $CD1d^{-/-}$ mice lacking both type I and type II NKT cells, but still possessing Treg cells, tumor growth was marginally suppressed compared with WT mice.[24,25] These results suggest that the loss of negative regulation by lacking type II NKT cells has stronger impact than that of antitumor effect by lacking type I NKT cells and that the lack of both NKT cell types plays some role in overcoming negative immune regulation.

In earlier reports of JTT as a cancer agent, studies were focused on immune-competent hosts. Thus, we hypothesized that JTT can exert antitumor effects in $CD1d^{-/-}$ mice, in which immunosuppression was partially relieved due to the lack of NKT cells.

In the present study, we showed the oral administration of JTT inhibited subcutaneously inoculated CT26 tumor growth in BALB-$CD1d^{-/-}$ mice. As expected, this did not occur in immune-competent WT BALB/c mice. JTT's antitumor effects in $CD1d^{-/-}$ mice were mediated by $CD8^+$ T cells. We also exclusively detected tumor-reactive cytotoxic T-lymphocytes (CTLs) in JTT-treated mice with well-controlled tumors. Additionally, we demonstrated a reduced number of $CD11b^+$ $Ly6G^+$ $Ly6C^{lo}$ polymorphonuclear (PMN)-MDSC and an increased number of degranulation marker $CD107a^+$ $CD8^+$ T cells. These were concomitantly observed in tumor-infiltrating lymphocytes (TILs) from CT26-bearing $CD1d^{-/-}$ mice treated with JTT, probably resulting in tumor growth inhibition. Our data indicate that, in the absence of NKT cells, $CD8^+$ T cell-mediated antitumor immunity may be augmented by JTT and that JTT is a useful adjuvant for combating cancers in conjunction with the strategy for removing immunosuppression.

Materials and Methods

Mice

We purchased inbred BALB/c mice from Charles River Japan (Tokyo, Japan). We purchased BALB/c $CD1d^{-/-}$ mice from Jackson Laboratory (Bar Harbor, ME). We established[26] and bred transgenic mice expressing TCR α and β genes of a murine CTL (cytotoxic T lymphocyte) clone RT1[27] (Tg-RT1) in our colony. These were specific for peptide P18-I10 restricted by D^d class I MHC (major histocompatibility complex) molecules, carrying the genetic background of BALB/c ($H\text{-}2D^d$). Mice were housed in groups of 5 in filter cages and maintained in a temperature-controlled, specific-pathogen-free animal facility. They were exposed to a constant 14-hour light–10-hour dark cycle, with free access to water and ad libitum access to food as indicated. We included female mice >6 weeks old

in all experiments. Experiments were approved by the Animal Care and Use Committee at Nippon Medical School (Tokyo, Japan). The Nippon Medical School has received external validation by the Japanese Association for Laboratory Animal Science. All animal experiments were performed appropriately in accordance with the guidelines for the Care and Use of Laboratory Animals, Nippon Medical School, Act on Welfare and Management of Animals and Standards related to the Care and Keeping and Reducing Pain of Laboratory Animals. A total of 3 to 6 mice/group were included in each experiment.

Diets Containing Japanese Kampo Medicine JTT

JTT, comprising 10 herbs (Table 1; http://mpdb.nibiohn.go.jp/stork/), was provided as a preservative-free pure powder by Tsumura & Co (Tokyo, Japan). As a control, we used MF diet (Oriental Yeast Co, Tokyo, Japan). Mice were fed a control diet or a control diet mixed with JTT (2.0%; designated JTT diet). Body weight and food intake in mice were measured 3 times a week using an electronic scale for 42 days (from 14 days prior to tumor inoculation to 28 days after tumor challenge). The amount of food intake in the JTT-diet group was almost equivalent to that in the control-diet group in $CD1d^{-/-}$ mice (Supplementary Figure 1C; available online). Interestingly, BALB/c mice consumed more of the JTT diet than the control diet (Supplementary Figure 1A). However, there were no differences in body weight between the control-diet and the JTT-diet groups of $CD1d^{-/-}$ or BALB/c mice (Supplementary Figure 1B and D).

Tumor Cell Lines

We purchased the N-nitro-N-methylurethane-induced BALB/c murine colon carcinoma CT26 cell line from the American Type Culture Collection (ATCC; Manassas, VA). The cell line was maintained in RPMI-1640 complete medium (ThermoFisher Scientific, Inc, Waltham, MA). The medium was supplemented with 10% fetal calf serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM); sodium pyruvate (1 mM), nonessential amino acids, HEPES (10 mM), MEM (minimum essential medium) vitamin solution, and 2-mercaptoethanol (5×10^{-5} M).

In Vivo Tumor Assay and Antibody Treatment

Prior to tumor challenge, BALB/c and $CD1d^{-/-}$ mice were fed the control or JTT diet for 14 days. We prepared a single-cell suspension of CT26 cells in phosphate-buffered saline (PBS; 5×10^4 cells in 200 μL) and then subcutaneously injected in the mice on day 0. The mice were fed the same diet for 28 days post-tumor inoculation. In a subset of experiments, $CD1d^{-/-}$ mice fed the JTT diet were injected intraperitoneally (ip) with 0.2 mg of anti-CD8 monoclonal antibody (clone, 2.43, cat. no. BE0061; Bio X Cell, West Lebanon, NH). Time points were as follows: 1 and 2 days prior to the inoculation with tumor cells and 4, 7, 10, and 14 days following the inoculation with tumor cells. The tumor area was measured 3 times a week using a caliper gauge. The measurements were calculated as tumor length (mm) × width (mm). When the tumor area was <100 mm^2 on day 28 post-tumor challenge, the mice were considered as "survived."[24]

Isolation of TILs

To isolate TILs, tumors were resected from mice and digested with 1 mg/mL collagenase (Roche Diagnostics GmbH, Mannheim, Germany) for 45 minutes at 37°C. The product was then gently crushed to homogenize the mass and then filtrated with a nylon mesh. To purify TILs, the leukocytes obtained through the latter process were further separated from contaminating tumor cells by lympholyte-M (CEDARLANE, Burlington, NC), according to the manufacturer's instructions.

Flow Cytometric Analysis

To determine the surface molecule expression of the cells, we performed flow cytometry using a FACSCanto II flow cytometer (Becton Dickinson Immunochemical Systems, Mountain View, CA). Specifically, to reduce nonspecific antibody binding to Fc receptors, isolated cells were incubated with 0.5 μg of anti-CD16/CD32 (clone 2.4G2, catalog number: 14-0161; eBioscience, Inc, San Diego, CA) at 4°C for 15 minutes. Subsequently, relevant antibodies were used to stain for 30 minutes at 4°C. The cells were then washed once with 2 mL of cold Dulbecco's phosphate buffer saline (DPBS; ThermoFisher Scientific) and analyzed. We used the following antibodies: APC/Cy7-conjugated anti-CD3 (clone 145-2C11, catalog number: 100329), FITC-conjugated anti-CD4 (clone RM4-5, catalog number: 100510), FITC-conjugated anti-CD8β (clone YTS156.7.7, catalog number: 126606), APC-conjugated anti-CD107a (clone 1D4B, catalog number: 121613), PECy7-conjugated anti-CD11b (clone M1/70, catalog number: 101215), APC/Cy7-conjugated anti-CD45 (clone 30-F11, catalog number: 103116), FITC-conjugated anti-Ly6G (clone 1A8, catalog number: 127605), and PE-conjugated anti-Ly6C (clone HK1.4, catalog number: 128007). We purchased all the antibodies from Biolegend (San Diego, CA). Dead cells were gated based on the uptake of propidium iodide.

For intracellular Foxp3 staining to detect $CD4^+$ $Foxp3^+$ T cells, following surface staining with anti-CD3 and anti-CD4 (in case of spleen cells) or with anti-CD3, anti-CD4, and

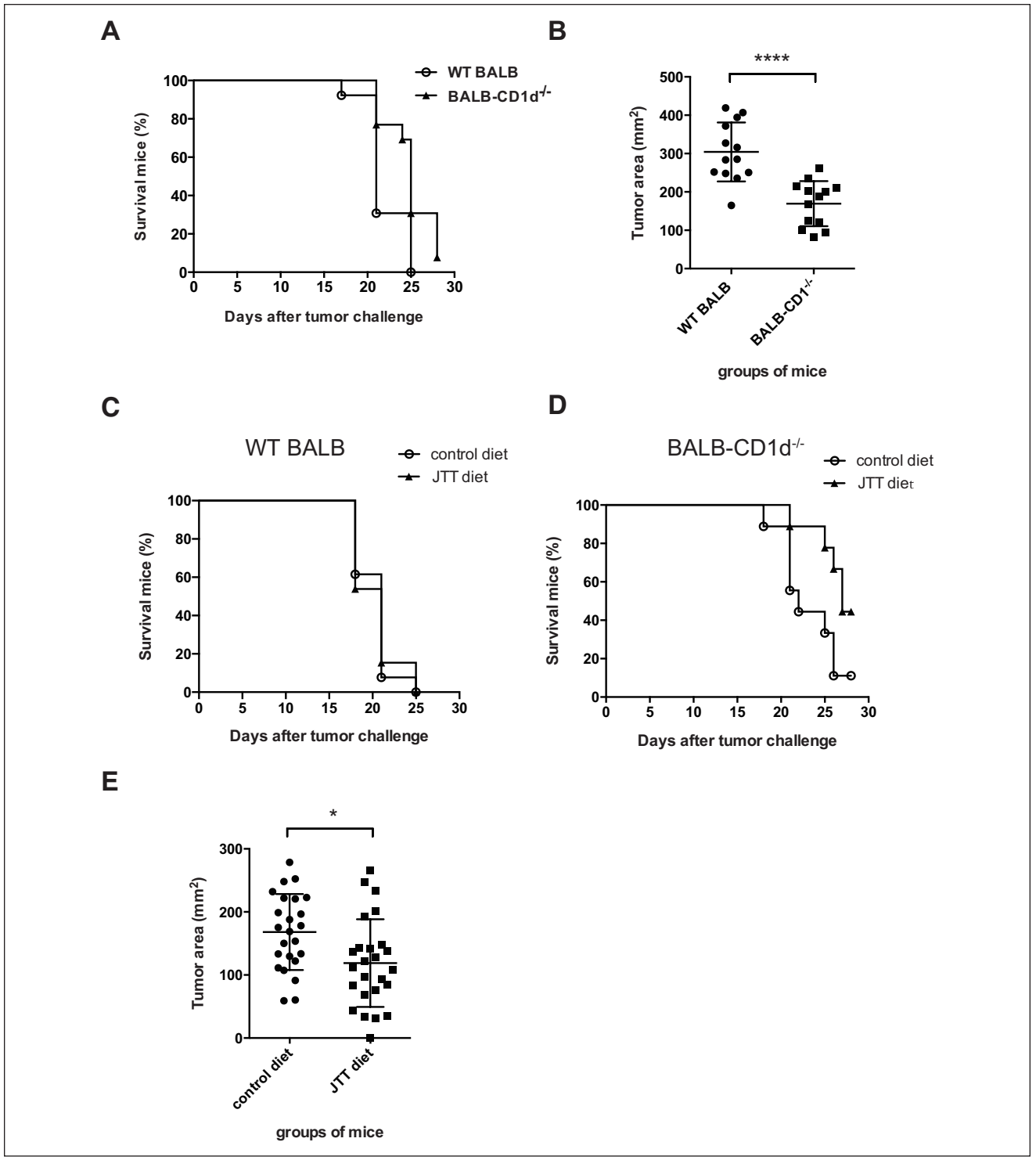

Figure 1. Oral administration of Juzentaihoto (JTT) inhibits tumor growth in CD1d$^{-/-}$ mice. We performed 3 (A, C) or 2 (D) independent experiments, and subsequently pooled the results. $P < .02$ in (A) by log-rank test between WT and CD1d$^{-/-}$ mice. $P < .03$ in (D) by log-rank test between CD1d$^{-/-}$ mice fed control versus JTT diet. The tumor area on day 28 following CT26 inoculation in mice is shown in (B) and (E). Each symbol corresponds to one data point. Data are expressed as mean ± standard deviation. *$P < .02$, ****$P < .0001$ (Mann-Whitney *U* test). In (E), we performed 5 independent experiments comparing the control (n = 24) versus the JTT (n = 25) diet groups, and subsequently pooled the results. Only one of the mice fed JTT diet completely rejected tumors, while none of the mice fed a control diet did.

anti-CD45 (in case of TILs), stained cells were washed once with 2 mL of cold DPBS. Cells were then fixed with 1 mL of Fixation/Permeabilization solution (eBioscience) at 4°C for 30 minutes. The fixed cells were then washed twice with 2 mL of 1× Permeabilization Buffer (eBioscience) and stained with 0.4 μg of PE-conjugated anti-Foxp3 (clone FJK-16s, catalog number: 12-5773-80; eBioscience) at 4°C for 30 minutes. The cells were washed twice with 2 mL of 1× Permeabilization Buffer.

For each sample, we acquired 3000 to 10 000 events. The data were subsequently analyzed using FlowJo software (version 9.3.1; Tree Star, Inc, Ashland, OR).

Functional MDSC Assay

We measured $Ly6G^+$ $Ly6C^{lo}$ PMN-MDSCs' suppressive function based on their ability to inhibit antigen-specific $CD8^+$ T cell proliferation.[28] We seeded CFSE-labeled splenocytes from Tg-RT1 mice at 2×10^5 cells/well in 96-round well plates in the presence of 1.6 μM peptide P18-I10 (Takara Bio Inc, Shiga, Japan). PMN-MDSCs were isolated from splenocytes derived from CT26-bearing $CD1d^{-/-}$ mice 28 days post-tumor inoculation using a mouse MDSC isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, isolated splenocytes were incubated with anti-Ly6G-biotin and anti-biotin microbeads. The cells were subsequently applied to a MACS column, which was placed in the magnetic field of a MACS separator. The magnetically labeled $Ly6G^+$ cells were retained within the column. After removing the column from the magnetic field, the magnetically retained Gr-1^{high} $Ly6G^+$ cells were eluted as the positively selected cell fraction. To check the purity of PMN-MDSCs, some of the eluted cells were stained with anti-Ly6G-FITC, anti-Ly6C-PE, anti-CD11b-PECy7, and anti-CD45-APCCy7. Among the $CD45^+$ $CD11b^+$ cells, approximately 90% were represented as the $Ly6G^+$ $Ly6C^{lo}$ population. We added purified PMN-MDSCs to CFSE-labeled Tg-RT1 splenoctyes at indicated ratios. After 96 hours post in vitro culture, we stained the cells with APC-conjugated anti-CD8α (clone 53-6.7, catalog number: 100711, Biolegend). To assess $CD8^+$ T cell proliferation, the cells were analyzed by flow cytometry.

In Vitro CTL Assay

We prepared single-cell suspensions of splenocytes from CT26-inoculated mice. After red blood cell lysis, we performed in vitro culture of 4×10^6 with 1×10^6 CT26 cells treated with 50 μg/mL mitomycin C in complete T cell media.[29] As a source of IL-2, rat T cell culture supplement, without ConA (Discovery Labware, Bedford, MA), was added to a final concentration of 10% on day 1. Cytotoxicity was assessed 7 days post in vitro culture, in a standard 4-hour ^{51}Cr-release assay with CT26 and BALB/c.3T3 ($H\text{-}2^d$) fibroblast transfectant. This contained selectable marker genes (Neo) as targets at various E:T ratios in triplicate wells. We calculated the percent lysis as previously described.[30]

Statistical Analysis

The data were analyzed using the following tests: Student's *t* test, nonparametric Mann-Whitney *U* test, and log-rank test. To this end, the GraphPad Prism 6 software (version 6.0d; GraphPad Software, La Jolla, CA) was used. We considered $P < .05$ a statistically significant difference.

Results

Oral Administration of JTT Inhibits Tumor Growth in $CD1d^{-/-}$ Mice That Lack NKT Cells

To test the hypothesis that JTT acts as an anticancer agent in a model in which immunosuppression was partially relieved due to lack of NKT cells, we investigated whether JTT could enhance antitumor immunity in $CD1d^{-/-}$ mice. To this end, WT BALB/c or BALB-$CD1d^{-/-}$ mice were fed a control or JTT diet for 2 weeks. Subsequently, mice were subcutaneously inoculated with 5×10^4 CT26 cells. The same diet was provided to these mice for up to 4 weeks after the tumor cell challenge. Similar to our previous report,[12] JTT did not have any impact on tumor growth in WT mice (Figure 1C). In contrast, we confirmed that the tumors grew significantly slower in $CD1d^{-/-}$ mice than in WT mice[24,25] (Figure 1A and B). Furthermore, as expected, we observed a significantly greater delay in tumor growth in $CD1d^{-/-}$ mice fed the JTT diet than those fed the control diet (Figure 1D and E). However, almost all JTT-treated mice failed to reject the tumors completely (Figure 1E). These observations suggest that JTT exerts antitumor effects in $CD1d^{-/-}$ mice but not in WT mice.

JTT Enhances CD8 T Cell Activities in TILs

$CD8^+$ TILs play pivotal roles in antitumor immune responses.[31] To this end, we measured the frequency of a degranulation marker $CD107a^+$ $CD8^+$ TILs in tumors derived from $CD1d^{-/-}$ mice fed control diet or JTT diet. Measurements were performed 28 days post-tumor challenge. As shown in Figure 2A and B, the proportion of $CD107a^+$ $CD8^+$ TILs in JTT mice was significantly higher than those in control mice. These observations suggest that an increased number of $CD107a^+$ $CD8^+$ TILs might enhance antitumor immunity in JTT-treated $CD1d^{-/-}$ mice.

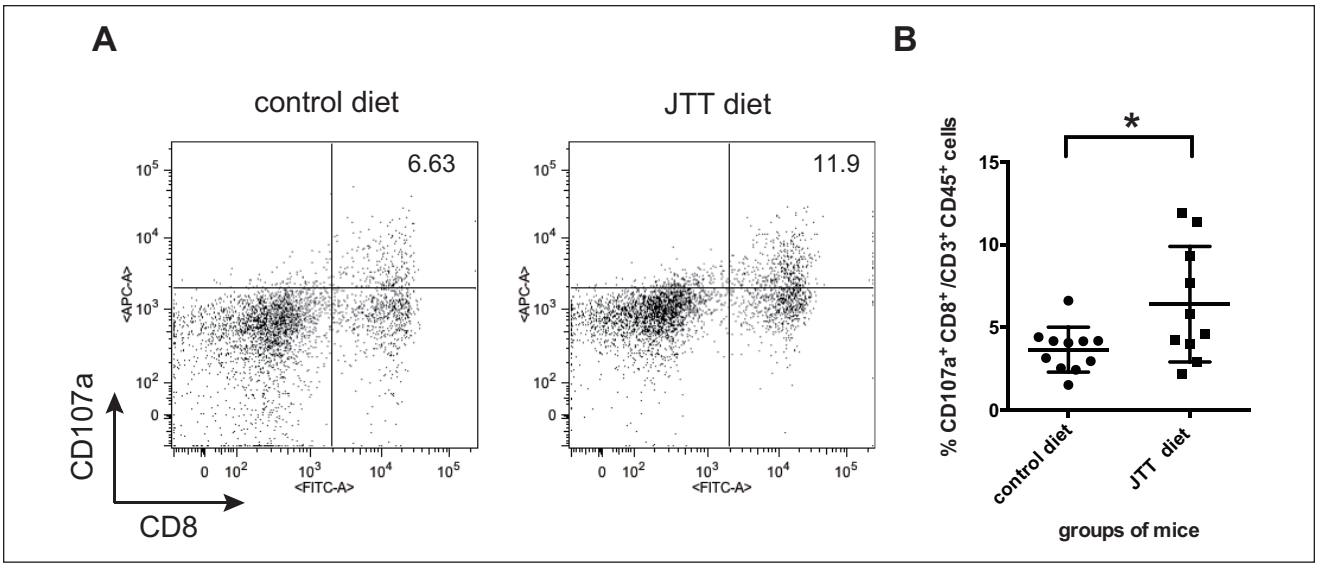

Figure 2. Juzentaihoto (JTT) enhances CD8 T cell activities in tumor-infiltrating lymphocytes (TILs). We determined, by flow cytometry, the proportions of $CD3^+$ $CD45^+$ $CD8\beta^+$ $CD107a^+$ cells. (A) Shows the representative flow data. Presented dot plots were gated on the $CD3^+$ $CD45^+$ population. (B) Shows the cumulative enumerations from 2 independent experiments. We used 5 or 6 mice per group. Each symbol corresponds to one data point. Data are expressed as mean ± standard deviation (n = 11 for control-diet group, n = 10 for JTT-diet group). *$P < .04$ (Student's *t* test with Welch's correction).

Tumor-Reactive CTLs Are Only Detected in CD1d$^{-/-}$ Mice Fed JTT Diet With Well-Controlled Tumors

Next, we analyzed the tumor-reactive CTLs in CT26-challenged $CD1d^{-/-}$ mice. After 28 days from tumor inoculation, we cultured with mitomycin C-treated CT26, spleen cells derived from $CD1d^{-/-}$ mice fed the control or JTT diet, as shown in Figure 3A. Culture was performed in vitro for 7 days. Subsequently, their cytolytic activity against CT26 was evaluated by ^{51}Cr release assay. Among the 11 tumor-bearing $CD1d^{-/-}$ mice fed a control diet tested, only one, with a tumor area <100 mm^2, had marginal lytic activity against CT26 (Figure 3B). In contrast, 4 out of 10 JTT-diet mice, with a tumor area <100 mm^2, had significant tumor-specific lytic activity (Figure 3C, and Figure 3D right) while the remaining 6 mice, with tumor size >100 mm^2, did not (Figure 3C, and Figure 3D left). These findings suggest that JTT may induce tumor-reactive CTLs in $CD1d^{-/-}$ mice where tumor growth was relatively suppressed.

Augmentation of Antitumor Effects Through JTT Requires CD8$^+$ T Cells

We also planned to elucidate whether $CD8^+$ T cells mediate the enhanced antitumor response in $CD1d^{-/-}$ mice fed the JTT diet. To this end, $CD8^+$ T cells were depleted in vivo. As shown in Figure 4, the anti-CD8 treatment in vivo abrogated antitumor effect in $CD1d^{-/-}$ mice fed JTT. These findings suggest a dependency on $CD8^+$ T cells for an enhanced antitumor immunity by JTT.

JTT Has No Impact on the Number of CD4$^+$ Treg Cells in CT26-Bearing CD1d$^{-/-}$ Mice

$CD4^+$ Treg cells are known to be key regulators of tumor immune surveillance.[14] The abrogation of such Treg cells by anti-CD25 antibodies enhanced antitumor immunity in both WT[22-24] and $CD1d^{-/-}$[25] mice subcutaneously inoculated with CT26 tumors. Therefore, in the present study, we measured the number of $CD4^+$ Treg cells in TILs and spleens in CT26-bearing $CD1d^{-/-}$ mice fed a control or JTT diet. Measurements were performed 28 days post-tumor challenge. When comparing the control-diet group and the JTT-diet group, we observed no difference in the number of $CD4^+$ Treg cells in TILs (Figure 5A and B) and in spleens (Figure 5C and D). These findings suggest that JTT has no impact on the number of Treg cells in CT26-bearing $CD1d^{-/-}$ mice.

JTT Reduces the Number of PMN-MDSCs in TILs

Because MDSCs also play crucial roles in the immunosuppression of tumor-bearing hosts,[15,32] we measured the frequency of MDSC in TILs and spleens derived from CT26-bearing $CD1d^{-/-}$ mice fed a control or JTT diet. Measurements were performed 28 days post-tumor challenge. In a recent study by

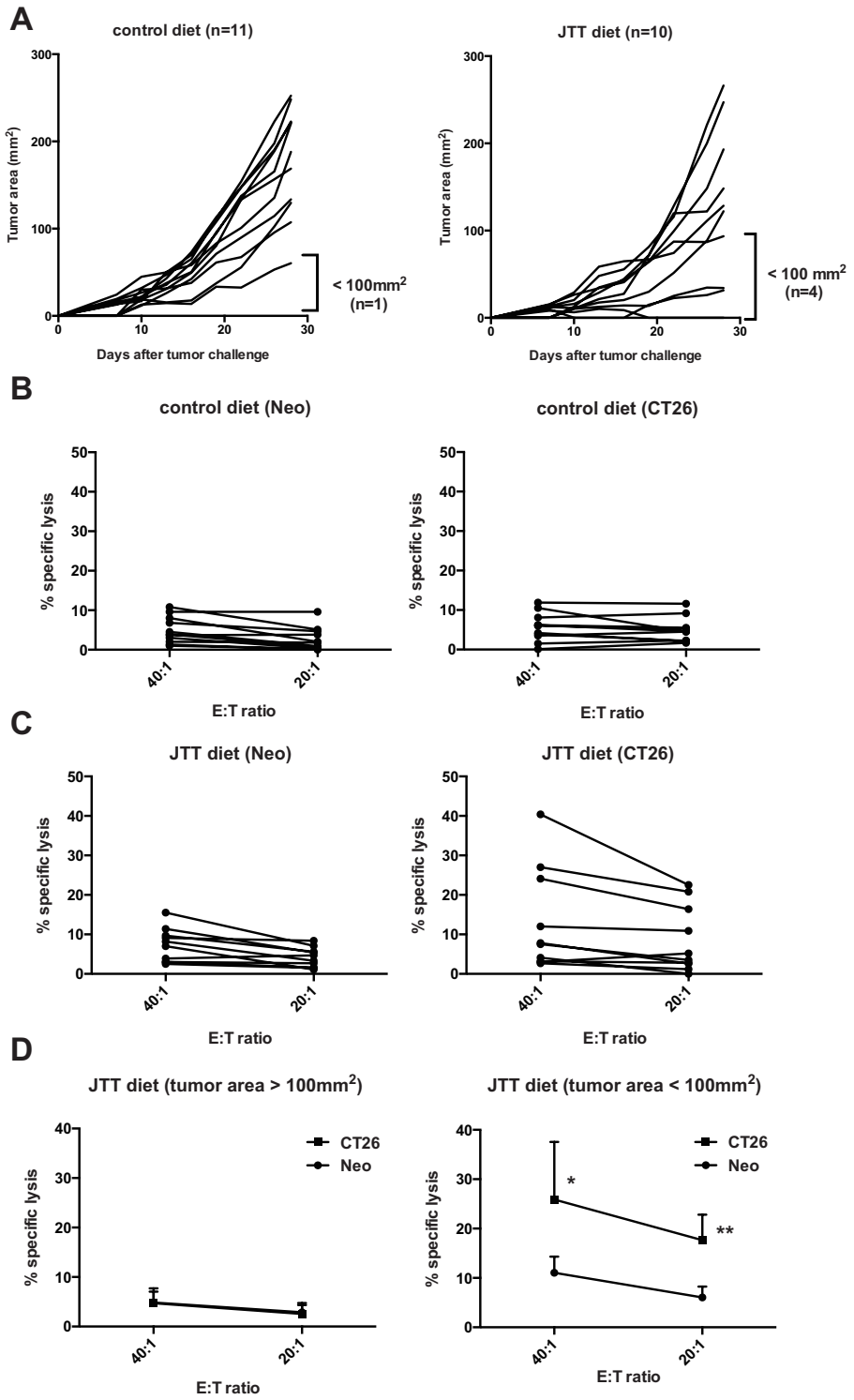

Figure 3. Tumor-reactive cytotoxic T-lymphocytes (CTLs) are only detected in Juzentaihoto (JTT)-treated CD1d$^{-/-}$ mice with well-controlled tumors. In (A), lines represent tumor growth of each individual mouse. We observed one mouse fed the control diet and 4 mice fed the JTT diet, with a tumor area <100 mm^2 on day 28 after tumor inoculation. As shown in (A), 28 days following tumor challenge, 4×10^6 splenocytes from CD1d$^{-/-}$ mice were cultured in vitro, with 1×10^6 CT26 treated with mitomycin C, for 7 days. Cytotoxicity against Neo and CT26 was examined using a 4-hour ^{51}Cr-release assay. Lines represent % specific lysis of individual mice (B, C). (D) Shows the average cytolytic activity of splenocytes from JTT-treated mice. The JTT-diet group with a tumor area >100 mm^2 contained 6 mice and JTT-diet group with a tumor area <100 mm^2 contained 4 mice. *$P < .05$, **$P < .007$ (Student's t test).

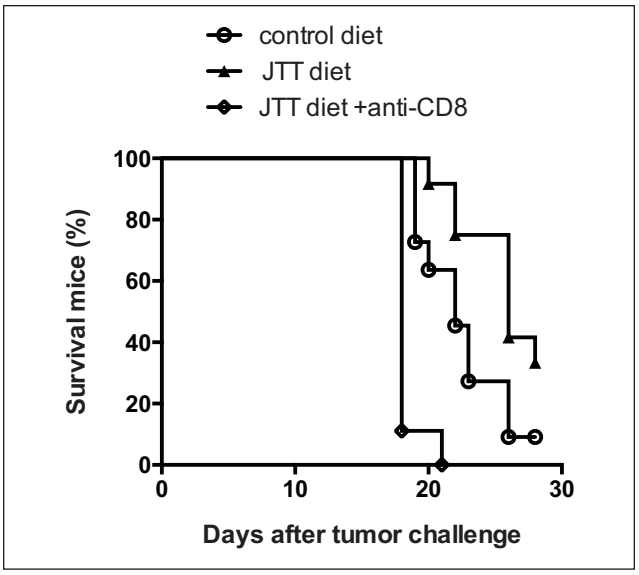

Figure 4. Antitumor immunity induced by Juzentaihoto (JTT) is mediated by $CD8^+$ T cells. We performed 2 independent experiments and pooled the results (n = 11 for control-diet group, n = 12 for JTT-diet group, and n = 9 for JTT diet + anti-CD8 group). $P < .03$ by log-rank test between the control and the JTT-diet group. $P < .0001$ by log-rank test between the control- or the JTT-diet group and JTT diet + anti-CD8 group.

Bronte et al,[33] it was shown that mouse MDSC are mainly divided into 2 types: $CD11b^+$ $Ly6G^+$ $Ly6C^{lo}$ PMN-MDSC; and $CD11b^+$ $Ly6G^-$ $Ly6C^{hi}$ M-MDSC. As shown in Figure 6A and B, compared with mice fed a control diet, there was a significant decrease of the frequency of PMN-MDSC in TILs in mice fed a JTT diet. In contrast, no significant differences were observed in the proportion of M-MDSC between control and JTT groups in TILs. Furthermore, there was no difference in the proportion of both types of MDSC between control and JTT groups in spleens (Figure 6C and D). To determine whether the reduced proportion of PMN-MDSCs in TILs was due to both the lack of NKT cells and JTT, we measured their frequency in TILs from JTT-treated WT mice. As expected, JTT had no impact on the frequency of PMN-MDSCs in TILs from WT mice (Supplementary Figure 2A and B). Interestingly, the frequency of splenic PMN-MDSCs from JTT-treated WT mice was significantly lower than that from control-diet mice (Supplementary Figure 2C and D). These observations suggest that the reduction of PMN-MDSC in TILs may contribute to the augmentation of antitumor immunity in $CD1d^{-/-}$ mice fed a JTT diet.

PMN-MDSC Derived From CT26-Bearing $CD1d^{-/-}$ Mice Fed the JTT Diet Are More Immunosuppressive

With the goal of elucidating PMN-MDSC's immunosuppressive function post CT26 inoculation, we isolated PMN-MDSC from spleens of CT26-bearing $CD1d^{-/-}$ mice as described in the Materials and Methods section. They were subsequently mixed with Tg-RT1 splenocytes in the presence of their epitope peptide P18-I10 for 4 days in vitro. As shown in Figure 6E and F, regardless of the diet, the addition of splenic PMN-MDSC derived from CT26-bearing mice determined a dose-dependent reduction of $CD8^+$ T cell proliferation. Interestingly, when compared with control-diet mice, the proliferation of $CD8^+$ T cells was strongly suppressed by PMN-MDSC in JTT-diet mice (Figure 6F).

Discussion

In the present study, we demonstrated that oral administration of JTT inhibited tumor growth in CD1d-restricted NKT cell-deficient $CD1d^{-/-}$ mice, but not in immune-competent BALB/c mice (Figure 1C and D). These findings highlight the critical role JTT plays in enhancing antitumor immunity in a tumor-bearing host when immunosuppression is partially relieved as a consequence of lacking NKT cells. In addition, $CD8^+$ T cells mediated JTT's antitumor immunity augmentation (Figure 4). However, in most $CD1d^{-/-}$ mice fed JTT, the tumors were not completely eradicated (Figure 1E). Moreover, we observed CTL reactive to CT26 exclusively in JTT-treated $CD1d^{-/-}$ mice with well-controlled tumors (Figure 3C, and Figure 3D right). Such findings suggest that JTT was not sufficient to maximize antitumor immunity in $CD1d^{-/-}$ mice. On the other hand, a previous study reported that, in the OVA-expressing EG7 murine tumor model, JTT enhanced tumor vaccine efficacy.[11] Additionally, Ishikawa et al[34] recently found that, in a B16 mouse melanoma model, JTT administration increased the inhibitory actions of anti-PD1 antibody against lung metastasis. Therefore, for the purpose of complete tumor rejection, the combination inhibiting the immunosuppressive pathway and tumor vaccine with JTT represents a promising strategy.

In a subcutaneous CT26 tumor model, $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells are predominant immunosuppressive cells. They play a role in suppressing the antitumor T cell effector function in both WT and $CD1d^{-/-}$ mice.[22-25] Consequently, we planned to elucidate whether JTT has any impact on $CD4^+$ Treg cells in $CD1d^{-/-}$ mice. We analyzed these cells in TILs and spleens derived from CT26-bearing $CD1d^{-/-}$ mice fed a control or JTT diet. Our results clearly indicated that there is no impact on the number of Treg cells by JTT, in both TILs and spleens from CT26-bearing $CD1d^{-/-}$ mice (Figure 5).

A recent study has recognized MDSC to be one of the major obstacles to natural antitumor immunity and to numerous immunotherapies.[35] In order to address this issue, we next measured the frequency of MDSC in TILs and spleens derived from CT26-bearing $CD1d^{-/-}$ mice fed control or JTT diet. Measurements were performed 28 days

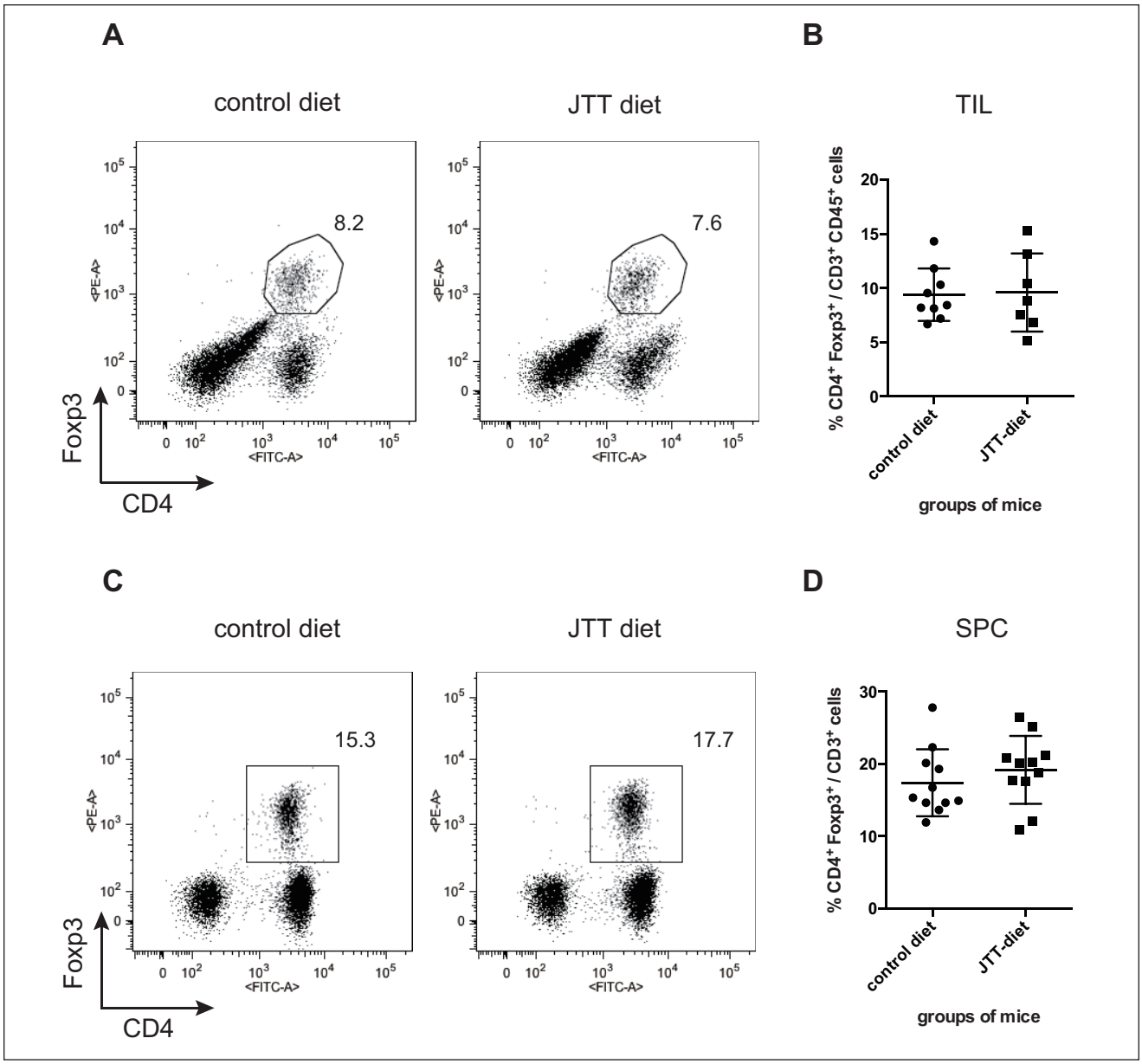

Figure 5. Juzentaihoto (JTT) has no impact on the frequency of $CD4^+$ $Foxp3^+$ Treg cells in tumor-infiltrating lymphocytes (TILs) and spleens from CT26-bearing $CD1d^{-/-}$ mice. Representative flow plots are shown as the percentage of $CD4^+$ $Foxp3^+$ cells in TILs (A) and spleen cells (C). We determined, by flow cytometry, the proportions of $CD4^+$ $Foxp3^+$ cells. For each group, we used 3 to 6 mice. We performed 2 independent experiments and pooled the results. Each symbol corresponds to one data point. The data are expressed as mean ± standard deviation.

post-tumor challenge. We observed that, when compared with control mice, the proportion of $CD11b^+$ $Ly6G^+$ $Ly6C^{lo}$ PMN-MDSC, but not $CD11b^+$ $Ly6G^-$ $Ly6C^{hi}$ M-MDSC, was significantly lower in TILs derived from JTT mice (Figure 6A and B). Furthermore, we observed a significant increase of the degranulation marker $CD107a^+$ $CD8^+$ TILs in JTT mice versus control mice (Figure 2). These data suggest that JTT's antitumor effect may be linked to a decrease in tumor-infiltrating PMN-MDSCs and an increase in $CD107a^+$ CTLs because of a significant tumor growth delay in JTT mice.

In the course of cancer progression, the production of various factors can be observed in tumor and stroma cells. Such factors are required to expand MDSCs via the promotion of myelopoiesis and the inhibition of differentiation, providing the necessary signals to endow their suppressive function.[36] Among these factors, vascular endothelial growth factor (VEGF) is known to be a chemoattractant for

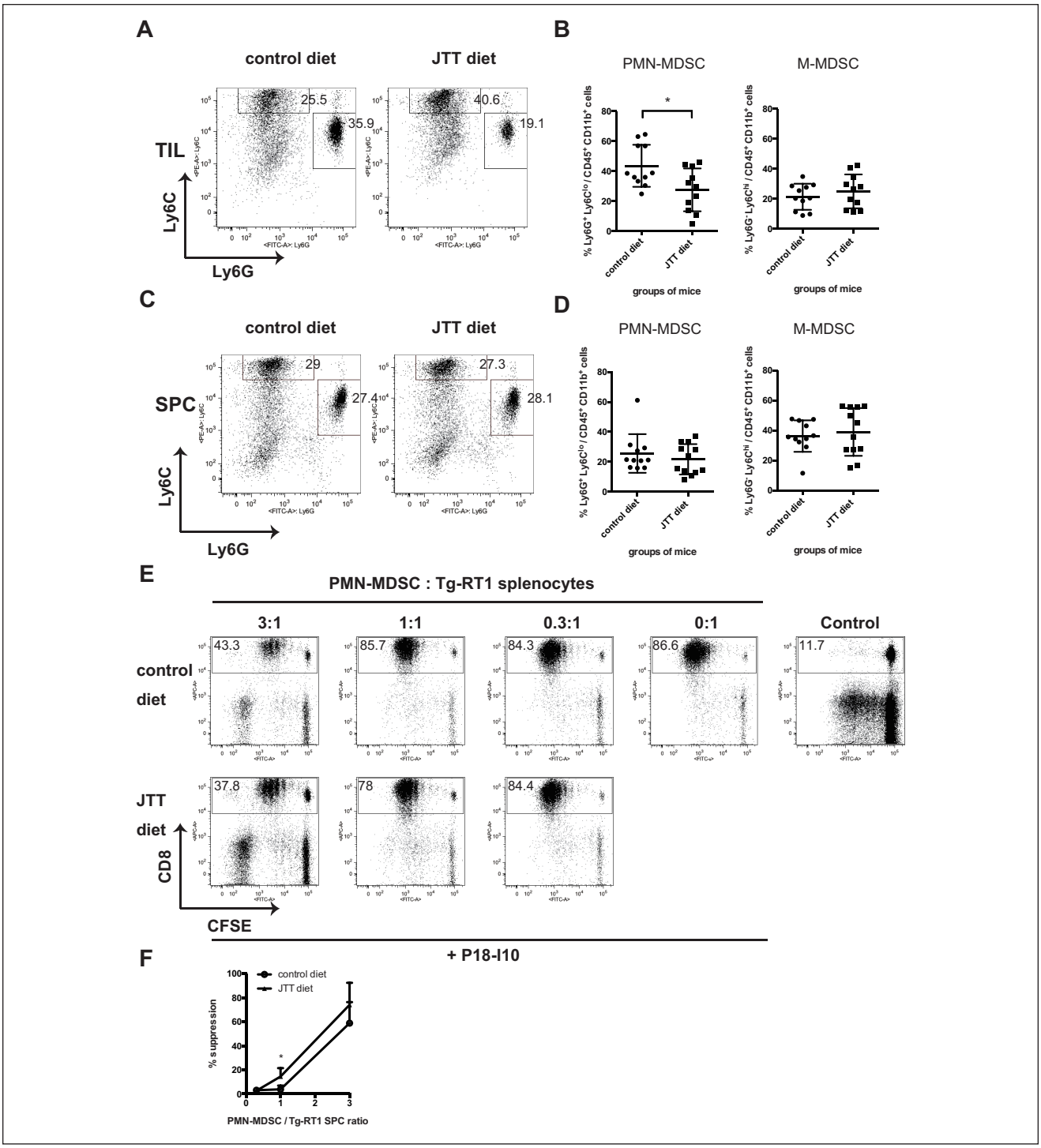

Figure 6. Juzentaihoto (JTT) reduces the proportion of polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs) in tumor-infiltrating lymphocytes (TILs) from CT26-bearing CD1d$^{-/-}$ mice, but enhances their immunosuppressive activity. We performed flow cytometry for Ly6G^{+} Ly6C^{lo} PMN-MDSC and Ly6G^{-} Ly6C^{hi} M-MDSC. (A) and (C) show representative flow data. Presented dot plots were gated on the CD45^{+} CD11b^{+} population. (B) and (D) show cumulative enumerations from 2 independent experiments. We used 5 to 6 mice per group for each experiment. Each symbol corresponds to one data point. Data are expressed as mean ± standard deviation (SD). *$P < .05$ (Mann-Whitney *U* test). Ly6G^{+} Ly6C^{lo} PMN-MDSC were isolated from pooled spleen cells derived from CT26-bearing CD1d$^{-/-}$ mice fed the control or JTT diet using MACS (as described in Materials and Methods). CFSE-labeled Tg-RT1 splenocytes were mixed with purified PMN-MDSC at indicated ratios in the presence of P18-I10 peptide (1.6 μM). Some splenocytes were cultured with medium only (Control). After 4 days, we used flow cytometry to analyze CD8^{+} T cells' proliferation. (E) Shows representative flow cytometry dot plots. Cumulative results ± SD are from 2 independent experiments (F: control diet, n = 4; JTT diet, n = 4). *$P < .04$ (Student's *t* test).

MDSCs[35] and is produced by MDSCs themselves.[37] A previous study has shown that JTT suppresses angiogenesis generated by B16 melanoma cells via VEGF inhibition.[38] Based on this knowledge, the reduction of PMN-MDSCs in TILs derived from JTT-treated mice may be explained by JTT's inhibition of VEGF. However, these findings warrant further investigation.

In recent preclinical and clinical studies, targeting MDSC represented a promising strategy to inhibit tumor growth and to prolong survival. However, due to extensive redundancy in MDSC inducers, depleting one mediator may be compensated by the presence of other mediators. As a result, while eliminating a single inducer may reduce the levels and suppressive potency of some MDSC, it is unlikely to eliminate all MDSC.[35] In line with this notion, while JTT-treated CD1d$^{-/-}$ mice had a significant reduction of tumor-infiltrating PMN-MDSC, we observed that JTT did not influence the proportion of tumor-infiltrating M-MDSC (Figure 6A and B). It also increased PMN-MDSC's immunosuppressive function (Figure 6E and F). These results suggest that JTT alone is incapable of fully eliminating MDSC and their immunosuppressive activity. This may explain why tumors are not eradicated in JTT-treated mice. Thus, we believe that additional targeting therapies against MDSC are needed in conjunction with JTT to eliminate MDSC and reduce their immunosuppressive function more effectively. A possible additional drug to be combined with JTT is represented by a chemotherapeutic drug, gemcitabine (GEM). GEM selectively decreased the number of MDSC in cancer-bearing hosts.[39] In addition, the results of a clinical trial on pancreatic cancer patients indicated GEM treatment caused a dramatic decrease of PMN-MDSC.[40] Furthermore, a study on advanced biliary tract cancer has shown that the tumor was successfully treated by combining GEM single-agent chemotherapy with JTT.[41] A marginal impact on overall survival of advanced pancreatic cancer patients was observed following treatment with JTT combined with GEP (GEM + 5-fluorouracil + cisplatin) therapy.[42] While MDSC's role was not described in these 2 studies, their observations suggest that using a combinatorial cancer immunotherapy (ie, JTT and GEM) may represent a strategy for a more effective MDSC targeting. Additional studies are required in order to clarify whether, in JTT-treated CD1d$^{-/-}$ mice, GEM perform pivotal roles in the enhanced antitumor immunity.

It should be noted that JTT was able to help restore physical strength postsurgery and reduce the adverse effects of anticancer drugs or radiation therapy.[3] Furthermore, studies have shown that JTT ameliorated cancer-induced anorexia and cachexia in CT26 tumor-bearing mouse model. Specifically, this was achieved by altering the production of IL-6, MCP-1, PYY, and GLP-1.[43] Additionally, JTT is considered a relatively safe, low-cost, and effective medicine that has been used for over 800 years in East Asia, including Japan.[1] It has been available under the Japanese National Health Insurance System since 1976. Treatment with JTT has been used even though there is no reliable statistical evidence from large-scale controlled studies to support it. Based on these findings, we believe that JTT, which is a relatively safe and cost-effective medicine, could be important for both augmenting antitumor immunity and providing supportive care for tumor-bearing hosts' management.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that JTT enhances CD8$^+$ T cell–mediated antitumor immunity in CD1d$^{-/-}$ mice lacking NKT cells. While further studies are needed to fully understand the underlying mechanism of JTT's anticancer effect, the present study provides a novel insight into JTT's use as antitumor adjuvant.

Acknowledgments

We would like to thank Ms Eri Koike for breeding the Tg-RT1 mice and Ms Atsuko Owaki for the detection of TCR expression in the Tg-RT1 mice. The authors would like to thank Enago (www.enago.jp) for the English language review.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The present study was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI, Japan (Grant Numbers 24501335 and 17K09328; received by ST).

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Supplemental Material

Supplemental material for this article is available online.

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