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Adeno-associated virus-clustered regularly interspaced short palindromic repeats/cas9-mediated ovarian cancer treatment targeting PD-L1

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Abstract

The response rate of antibody therapy targeting immune checkpoint molecules in ovarian cancer is insufficient. This study aimed to develop a novel gene immunotherapy model targeting programmed death ligand 1 (PD-L1) in vivo in ovarian cancer using adeno-associated virus (AAV)-clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 and investigate its efficacy. In vitro, we produced PD-L1-AAV particles to knock out PD-L1. PD-L1-AAV particles were transduced into the murine ovarian cancer cell line ID8. PD-L1 expression at the cellular level was significantly decreased following treatment with PD-L1-AAV particles compared with control-AAV particles. In the peritoneal dissemination model, the survival time was significantly longer in the PD-L1-AAV particles intraperitoneally injected group than that in the control group. Furthermore, intratumoral lymphocyte recruitment was analyzed by immunohistochemistry, and the number of intratumoral $CD4^+$ and $CD8^+$ T cells was significantly higher, whereas that of $Foxp3^+$ Treg cells was significantly lower in the PD-L1-AAV particles injected group than in the control group. No severe adverse events in normal organs, such as the lungs, spleen, liver, and kidney, were observed. These results suggest that PD-L1-targeted therapy by genome editing using AAV-CRISPR/Cas9 is a novel gene-immune therapeutic strategy for ovarian cancer.

Keywords PD-L1, AAV-CRISPR/Cas9, Ovarian cancer, Genome editing

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Introduction

Ovarian cancer has the highest mortality rate among gynecological malignancies. Approximately half of all cases are diagnosed at an advanced stage with peritoneal or distal metastases. The standard treatment for advanced ovarian cancer includes debulking surgery and platinum–taxane chemotherapy. Recently, a complete cure has been reported to be possible in some cases with the use of anti-vascular endothelial growth factor antibodies and poly (ADP-ribose) polymerase inhibitors [1, 2]. However, the long-term prognosis of patients with ovarian cancer has not improved satisfactorily over the past decade, and the 5-year relative survival rate for ovarian cancer is only 49% [3]. Therefore, developing novel therapeutic strategies is urgently needed to further prolong the survival of patients with this disease.

Recently, immune checkpoint inhibitors (ICIs) have been reported to be effective in treating various cancers, with single-agent response rates of 9–30% [4, 5]. However, the response rate of anti-programmed death 1 (PD-1) and programmed death ligand 1 (PD-L1) antibodies for recurrent ovarian cancer has been reported to be even lower (5.9–15%) [6]. The response rate to ICIs in patients with ovarian cancer was not satisfactory; hence, it is necessary to develop novel methods of inhibition to enhance therapeutic efficacy. Additionally, the immune-related adverse events (irAEs) of ICIs, occurring in approximately 70% of patients [7, 8], must be reduced.

In our previous study, we focused on the PD-L1/PD-1 pathway, a molecule playing a role in inducing tumor immune tolerance, and established the murine ovarian cancer cell line ID8 with the complete absence of the PD-L1 gene using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system. In a mouse peritoneal dissemination model, the PD-L1-KO ID8-inoculated mice showed a significantly longer survival time than the control group. Additionally, intratumoral $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and $CD11c^+$ M1 macrophages significantly increased, regulatory T cells significantly decreased, and tumor weight significantly reduced [9]. These findings suggest that gene immunotherapy targeting PD-L1 in tumor cells using the CRISPR/Cas9 system may be a new therapeutic strategy to replace these antibody therapies for ovarian cancer. However, since these genes have been knocked out at the cellular level in previous studies, the problem of how to perform in vivo gene editing using CRISPR/Cas9 has not yet been solved for clinical application. Therefore, we focused on the adeno-associated virus (AAV)-CRISPR/Cas9 system to directly delete PD-L1 expressed in ovarian cancer.

AAV is a non-pathogenic parvovirus that can infect both dividing and non-dividing cells of various tissues, mediates long-term and robust gene expression, is less immunogenic, and has no known human diseases associated with its infection, making it a very effective vector for in vivo gene transfer and editing [10]. In particular, AAV serotype 2 (AAV2) has been well studied and widely used. Early studies showed that AAV2 has the greatest tropism for liver and skeletal muscle and that the AAV genome persists for at least one year and continue to mediate high levels of expression [11]. However, since large-scale production of AAV is still difficult [10], it is necessary to develop methods to increase AAV production efficiency and establish methods to make it work efficiently.

AAV-based CRISPR/Cas9 delivery for in vivo genome editing has made exciting progress in numerous disease models, including blood disorders [12], liver diseases [13], muscular diseases [14, 15], motor neuron disease [16], and cancer [17–19]. However, no treatment strategies for ovarian cancer have been reported to target PD-L1 on tumors using the AAV-CRISPR/Cas9 system. Thus, this study aimed to develop a novel gene immunotherapy model targeting PD-L1 in ovarian cancer by taking advantage of the cancer-directed nature of AAV as a method to reduce the severe irAEs reported in ICIs and efficiently exert tumor suppressive effects.

Materials and methods

Reagents and antibodies

The following monoclonal antibodies (mAb) was used: rabbit anti-mouse CD4 mAb (cat. no. #25229; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-mouse CD8α mAb (cat. no. #98941; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-mouse Foxp3 mAb (cat. no. #12653; Cell Signaling Technology, Danvers, MA, USA), and rabbit anti-mouse PD-L1mAb (cat. no. #64988; Cell Signaling Technology, Danvers, MA, USA) were used for immunofluorescence analyses.

Cell line and culture

The mouse ovarian carcinoma cell line ID8 was kindly gifted by Dr. Kathy Roby (University of Kansas Medical Center, Kansas City, KS, USA). The cell line was cultured in minimum essential medium alpha (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), penicillin, streptomycin, and amphotericin B (Life Technologies).

Mice

Specific pathogen-free 8-week-old female C57BL/6 (B6) mice were purchased from CLEA Japan, Inc., Tokyo, Japan. A total of 32 mice were used for the in vivo experiments in the overall survival and the tumor progression model. The overall survival model used eight mice for the intraperitoneal injection of PD-L1-AAV particles and

eight mice for the control group. The tumor progression model used a total of eight mice for the intraperitoneal injection of PD-L1-AAV particles and eight mice for the control group. All animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University (approval number 964 and 1132).

Construction of the plasmid vector

sgRNAs targeted to Cd274 (Pdl1) exon 3 (NC_000085.6) were designed using the open-access software programs NCBI Gene (https://www.ncbi.nlm.nih.gov/gene) and CRISPRdirect (https://crispr.dbcls.jp). sgRNAs were chosen from previous reports based on the significant effects found [9]. The AAVpro CRISPR/SaCas9 Helper Free System (AAV2) was purchased from Takara Bio Inc (Mountain View, CA, USA). The sgRNA (5′-GGTCCAGCTCC CGTTCTACA-3′), targeting exon 3 of Pdl1, was cloned into the pAAV-Guide-it-Down vector. The plasmid vector (pAAV-Guide-it-Down-PD-L1) was sequenced to confirm successful ligation. Transfection was performed using pAAV-Guide-it-Down-PD-L1 or pAAV-Guide-it-Up packaging pRC2-mi342 and pHelper vectors with calcium phosphate. After 6 h, the transfection medium was replaced with fresh Dulbecco's Modified Eagle Medium containing 2% fetal bovine serum and 1% antibiotic antimycotic solution and incubated at 37℃. Cells were collected 72 h after transfection, and AAV particles were harvested using AAV extraction solutions A and B (Takara Bio Inc) according to the manufacturer's instructions and stored at − 80℃ for further use. AAV2 vectors were purified using the AAVpro Purification Kit (Takara Bio Inc) according to the previous reports [13, 20, 21] and the manufacturer's instructions. After purification, the virus titer (virus genomic titer) was measured by real-time polymerase chain reaction using the ITR domain as the target region with the AAVpro Titration Kit (Takara Bio Inc) following the manufacturer's instructions. All experiments with recombinant DNA technology were approved by the DNA Recombination Experiment Committee of Wakayama Medical University (approval numbers 29–34 and 2021-71).

Western blot analysis

For the in vitro experiments, cells were seeded in 6-well plates (2.0×10^5 cells per well). After a 6-h incubation, the cells were transduced for 72 h with purified AAV2-Up and PD-L1-AAV2-Down particles (PD-L1-AAV particles) at a 1:1 ratio (virus genomic titer) for PD-L1 modification of ID8 cells using an MOI of 1000. The growth medium was removed and the cells were stimulated with or without 20 ng/ml of recombinant mouse IFN-γ for 24 h. The cells were then lysed in lysis buffer [1% Triton/PBS (-), containing protease inhibitor cocktail tablets (cOmplete, Mini, EDTA-free, Merck, Darmstadt, Germany)/PBS (-), pH 7.5]. For the in vivo experiments, the tumors isolated from the mice injected with PD-L1-AAV or control AAV particles were homogenized in 10% RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (Cell Signaling Technology, Inc., Danvers, MA, USA), 0.1% sodium dodecyl sulfate (NACALAI TESQUE, INC., Kyoto, Japan), and protease inhibitor cocktail tablets (Merck)/PBS (-), pH7.5. A total of 14 μg of protein was separated by electrophoresis using 12.0% polyacrylamide e-PAGEL® gels (ATTO Corporation, Tokyo, Japan) and transferred to polyvinylidene fluoride membranes (ATTO corporation). The membranes were incubated with a primary antibody against PD-L1 (dilution 1:1500, cat. no. AF1019; R&D Systems, Minneapolis, MN, USA) or GAPDH (dilution 1:5000, cat. no. MAB374; Merck, Darmstadt, Germany) at 4℃ overnight. After incubation with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h, specific proteins were visualized by chemiluminescence detection (EZ West Lumi, ATTO corporation). Signals were visualized using a LuminoGraph image analyzer (ATTO corporation). To determine the densities of PD-L1-positive dots, image files were analyzed using ImageJ 1.50i software (NIH).

Tumor formation in vivo

The effects of PD-L1 KO on tumor progression and survival were evaluated using a mouse ovarian cancer peritoneal dissemination model, as reported previously [9]. ID8 cells (1.5×10^6) were injected into the abdominal cavities of syngeneic mice, and PD-L1-AAV particles (MOI of 35) were injected repeatedly every week for 9 times on days 3, 10, 17, 24, 31, 38, 45, 52 and 59 into the abdominal cavities. Survival time and disseminated tumor weight were evaluated at the indicated time intervals after inoculation. For the overall survival model, after intraperitoneal inoculation of ID8 cells, PD-L1-AAV particles were injected intraperitoneally and survival time was evaluated. For the tumor progression model, after intraperitoneal inoculation of ID8 cells, PD-L1-AAV particles were injected intraperitoneally, and the disseminated tumor weight was measured on day 74, when the mouse body weight had increased. These disseminated tumors on the peritoneum and omentum were resected after laparotomy, and tumor weight was measured. To monitor the mice, we determined their weight gain every day. In addition, we confirmed that the mice had no difficulty while ingesting food or water, no moribund symptoms, and no abnormal appearance over a prolonged period or visible indications of recovery. The mice were euthanized before reaching a moribund state. The humane endpoint included food and water intake difficulties, moribund symptoms, abnormal appearance over a prolonged period with no visible signs of recovery, weight loss (20% or more over several days),

and a marked increase in tumor size (10% or more of body weight). Laboratory animals were euthanized using a bell jar system [22]. Isoflurane (cat. no. 099-06571; FUJIFILM Wako Pure Chemical Corporation, Japan) was administered at a 5% maintenance dosage for 2–3 min. Exposure to isoflurane lasted up to 1 min after respiratory arrest. Changes in body weight were measured, and the side effects of AAV particles on normal organs, such as the lungs, liver, spleen, and kidneys, were assessed using hematoxylin and eosin staining.

Immunohistochemical analyses

Immunohistochemical staining was outsourced to Morphotechnology Co., Ltd. (Hokkaido, Japan). Briefly, tumor samples were fixed in 4% paraformaldehyde, and paraffin-embedded specimens were cut into 4 μm thick sections. Deparaffinized sections were stained with rabbit anti-mouse CD4 (1:50), rabbit anti-mouse CD8 (1:300), and rabbit anti-mouse Foxp3 (1:100) antibodies. The number of $CD4^+$ T cells, $CD8^+$ T cells, and $Foxp3^+$ regulatory T cells at the tumor site was counted on 15 randomly selected visual fields at 400× magnification. The average of the five selected microscopic fields was calculated.

To further examine the effects on normal organs, such as the lungs, liver, spleen, and kidneys, the number of PD-L1 positive cells was evaluated. Each organ was fixed in 4% paraformaldehyde, and paraffin-embedded specimens were cut into 4 μm thick sections. Deparaffinized sections were stained with rabbit anti-mouse PD-L1 (1:200) antibodies. The number of PD-L1-positive cells in normal organs was calculated by averaging the number at 400x magnification for 10 randomly selected fields of view in 8 mice from each group.

Table 1 Sequences of primers used for real-time RT-PCR

Transcript	Sequence	GenBank accession number
Ifng	(Fw.) 5′- CGGCACAGTCATTGAAAGCCTA -3′ (Rv.) 5′- GTTGCTGATGGCCTGATTGTC -3′	NM_008337.4
Il2	(Fw.) 5′- CCCAGGATGCTCACCTTCA -3′ (Rv.) 5′- CCGCAGAGGTCCAAGTTCA -3′	NM_008366.3
Il12a	(Fw.) 5′- TGTCTTAGCCAGTCCCGAAACC -3′ (Rv.) 5′- TCTTCATGATCGATGTCTTCAGCAG -3′	NM_008351.2
Cxcl9	(Fw.) 5′- CCGAGGCACGATCCACTACA -3′ (Rv.) 5′- AGTCCGGATCTAGGCAGGTTTG -3′	NM_008599.4
Actb	(Fw.) 5′- CATCCGTAAAGACCTCTATGC-CAAC -3′ (Rv.) 5′- ATGGAGCCACCGATCCACA -3′	NM_007393.5

fng: interferon-γ, IL: interleukin, CXCL: Chemokine (C-X-C motif) ligand, ACTB: beta-actin

Real-time RT-PCR

Total RNA was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). Total RNA (1 μg) was reverse transcribed into cDNA at 37 °C for 15 min using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio Inc). The resulting cDNA was then subjected to real-time PCR analysis using the SYBR Premix Ex Taq II kit (Takara Bio Inc) with gene-specific primer sets (Table 1).

The amplification and detection of mRNA were performed using the Thermal Cycler Dice Real Time System (Takara Bio Inc) based on the manufacturer's instructions. The relative quantity of target gene expression relative to the β-actin gene was measured using the comparative Ct method as described previously [9].

Enzyme-linked immunosorbent assay (ELISA)

The tumors isolated from the mice injected with PD-L1-AAV or control AAV particles were homogenized, and the supernatant was subjected to ELISA. Interferon-γ (IFN-γ), Interleukin (IL)-6, IL-10, and vascular endothelial growth factor (VEGF) levels were measured with a commercially available ELISA kit (R&D systems) according to the manufacturer's instructions. The lower detection limit of IFN-γ, IL-6, IL-10, and VEGF were 37 pg/ml,15.6 pg/ml, 15.6 pg/ml, and 15.6 pg/ml, respectively. Total protein in each supernatant was measured with a commercially available kit (BCA Protein Assay Kit; Pierce). Data were expressed as cytokine per protein (pg/ml) for each sample.

Statistical analyses

All parameters examined in this study were presented as mean and standard error (SE). The significance of differences was evaluated using an unpaired Student's t-test. Survival was analyzed using Kaplan–Meier curves and the log-rank test. Statistical analyses were performed using JMP Pro, version 13 (SAS Institute Inc., Cary, NC, USA). $p < 0.05$ was considered statistically significant.

Results

Effect of PD-L1-AAV particles on PD-L1 expression

We successfully generated purified AAV2-Up and PD-L1-AAV2-Down particles (PD-L1-AAV particles) (Fig. 1).

PD-L1-specific gRNA/Cas9 systems were constructed using gRNA-targeted to *Cd274 (Pdl1)* exon 3, which were delivered using AAV vectors, and their ability to suppress the PD-L1 gene in ID8 cells was assessed. To confirm knockdown efficiency, we examined PD-L1 expression when cells were treated with PD-L1-AAV or control-AAV particles by western blot analysis. PD-L1 expression at the cellular level was significantly decreased following treatment with PD-L1-AAV particles compared with control-AAV particles. The knockdown efficiency was 26.5% (Fig. 2A, B, Supplementary Fig. 1, 2).

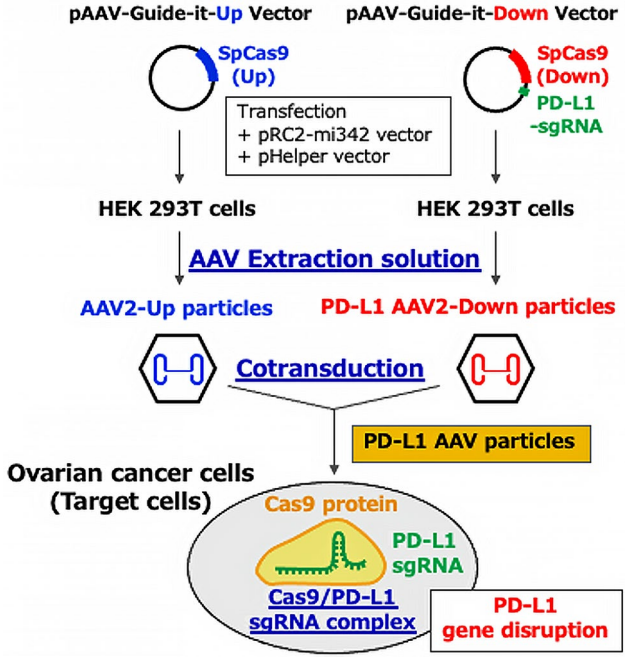

Fig. 1 PD-L1-AAV particles targeting PD-L1 exon 3 were designed and engineered as described in previous experiments. Thereafter, we established an AAV-CRISPR/Cas9 system to deliver genome editing of PD-L1 on tumors in vivo. PD-L1-specific gRNA/Cas9 systems were constructed using gRNA targeted to Cd274 (Pdl1) exon 3, which were delivered using AAV vectors, and their ability to suppress the PD-L1 gene in ID8 cells was assessed. These control AAV and PD-L1-AAV particles were used in subsequent experiments. (Modified from references number 41)

Effect of PD-L1-AAV particles on tumor progression and survival

ID8 cells were injected intraperitoneally into syngeneic mice, and then PD-L1-AAV or control AAV particles were injected intraperitoneally repeatedly every week for 9 times on days 3, 10, 17, 24, 31, 38, 45, 52, and 59 to evaluate the treatment effect of PD-L1-AAV particles on tumor progression and survival in vivo (Fig. 3A, B). Survival times were significantly longer in the PD-L1-AAV particles intraperitoneally injected group than in the control group (Fig. 3C). The extent of intraperitoneal tumor dissemination was evaluated 74 days after the injection of ID8 cells. Tumor weights were significantly lower in the PD-L1-AAV particles intraperitoneally injected group than in the control group (Fig. 3D, E). To confirm the knockdown efficiency in vivo, we examined the expression of PD-L1 protein in tumors isolated from PD-L1-AAV or control-AAV particle-treated mice by western blot analysis (Fig. 4A, Supplementary Fig. 3). Intratumoral expression of PD-L1 was markedly decreased in PD-L1-AAV particle-treated mice compared with control-AAV particle-treated mice (Fig. 4B).

Effect of PD-L1 AAV particles on intratumoral lymphocyte infiltration

Immunohistochemical analyses and tumor-infiltrating lymphocyte profiling were performed to elucidate the mechanism by which intraperitoneal injection of PD-L1 AAV particles for 9 times on days 3, 10, 17, 24, 31, 38, 45, 52 and 59 was repeated every week that suppressed ovarian cancer progression. The number of intratumoral $CD4^+$ T and $CD8^+$ T cells was significantly higher in the PD-L1-AAV particles intraperitoneally injected group than in the control group (Fig. 5A, B). In contrast, the number of $Foxp3^+$ regulatory T cells was significantly lower in the PD-L1-AAV particles intraperitoneally injected group than in the control group (Fig. 5C).

Cytokine and chemokine levels in disseminated tumors

To identify the molecular mechanisms, we evaluated the intratumoral mRNA expression of cytokines and

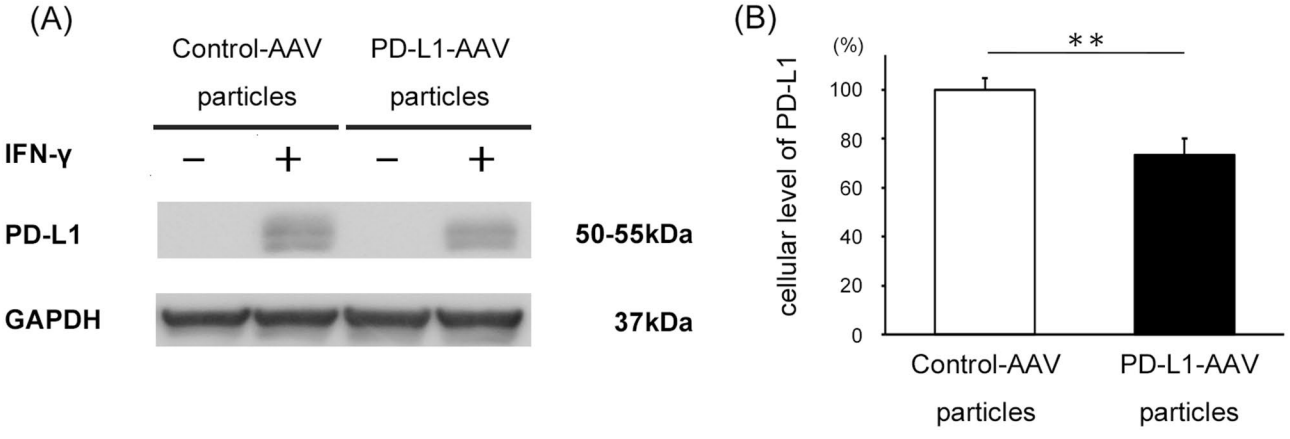

Fig. 2 PD-L1-AAV particles reduced PD-L1 expression. PD-L1 expression at the cellular level was determined by western blot analysis. GAPDH was used as a loading control (**A**). Western blot results were analyzed using the relative quantification (RQ) value, and the knockdown efficiency was calculated as (1-RQ) ×100 (%). All values are presented as the mean ± SE of three independent experiments. Statistical significance was determined using an unpaired Student's t-test. ** $p < 0.01$ (**B**)

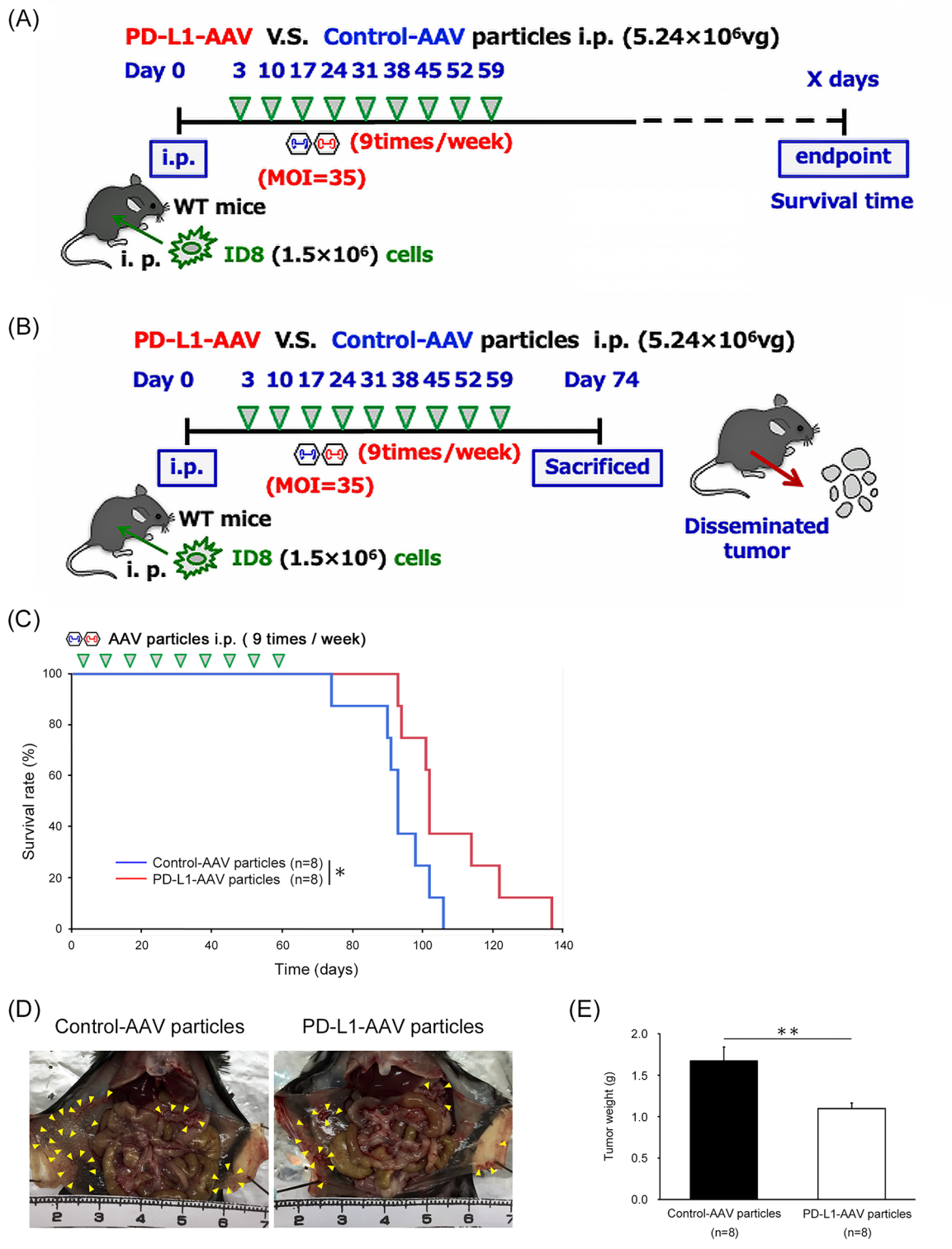

Fig. 3 Effect of PD-L1 AAV particles on mouse survival and tumor dissemination. Schematic presentation of the intraperitoneal injection of PD-L1-AAV particles given on days 3, 10, 17, 24, 31, 38, 45, 52, and 59 (9 times, repeated every week) in a mouse ovarian cancer peritoneal dissemination model on survival in vivo (**A**). Tumor progression (**B**). (**C**) Kaplan–Meier curves and the log-rank test in an overall survival analysis of PD-L1-AAV and control AAV particles (each group, $n=8$). * $p<0.05$. (**D**) Photographs of the extent of peritoneal dissemination 74 days after the injection of PD-L1-AAV and control AAV particles. Yellow arrowheads indicate disseminated tumors on the peritoneum and omentum. Representative results from eight independent mice in each group are shown. (**E**) Intraperitoneal tumor weights 74 days after the injection of PD-L1-AAV and control AAV particles. All values are presented as mean ± SE ($n=8$). Statistical significance was determined using an unpaired Student's t-test. ** $p<0.01$

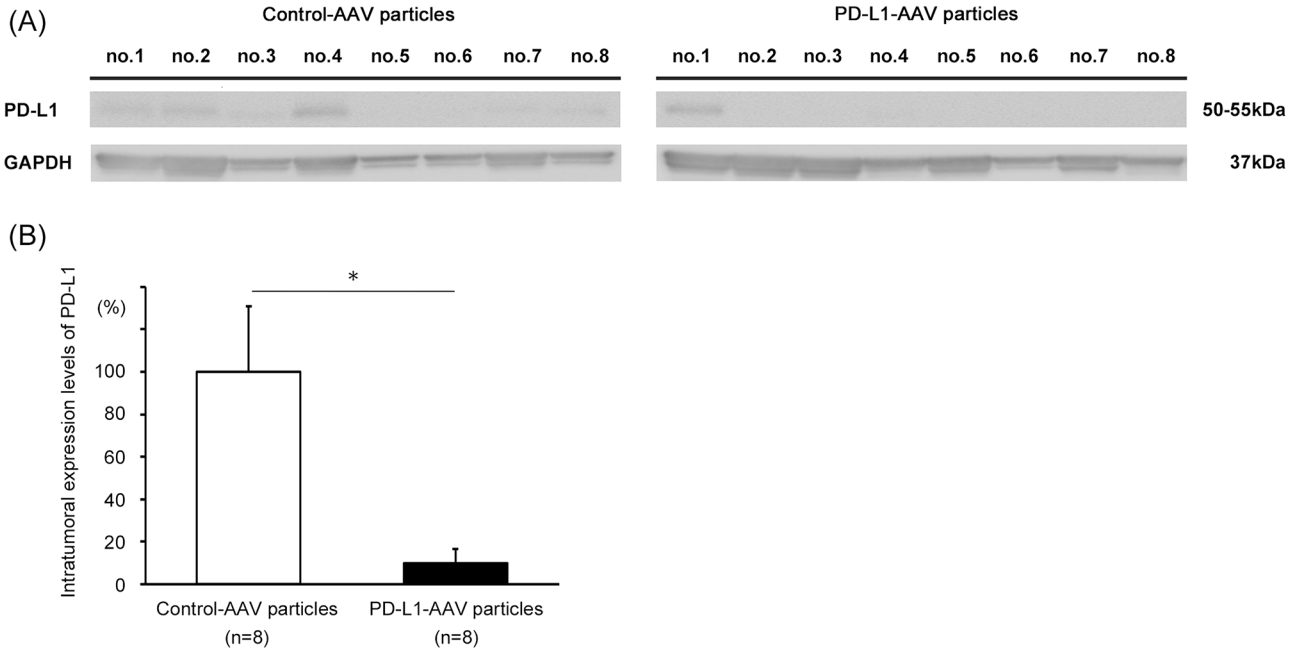

Fig. 4 PD-L1-AAV particles reduced PD-L1 expression in vivo. (4 A) PD-L1 expression in the isolated tumors was measured by western blot analysis (each group, $n=8$). GAPDH was used as a loading control. (4B) Western blot results were analyzed using the relative quantification (RQ) value. All values are presented as the mean ± SE (each group, $n=8$). Statistical significance was determined using the unpaired Student's t-test. * $p<0.05$

chemokines using real-time RT-PCR. The intratumoral mRNA expression of IFN-γ, IL-2, IL-12a, and chemokine (C-X-C motif) ligand (CXCL) 9 in the tumor microenvironment tended to be higher in the PD-L1-AAV particle-injected group compared with the control group. However, there were no significant differences between the two groups (Supplementary Fig. 4).

In addition, we evaluated the intratumoral protein concentration of cytokines using ELISA. The levels of IL-10 and VEGF in the tumor microenvironment were significantly lower in the PD-L1-AAV particles intraperitoneally injected group than in the control group (Fig. 6). Moreover, the levels of IFN-γ tended to be higher, and the levels of IL-6 tend to be lower in the PD-L1-AAV particle-injected group compared with the control group, although these were no significant differences between the two groups.

Adverse effects of PD-L1 AAV particles on normal organs

Histopathological analysis using hematoxylin and eosin staining revealed no structural abnormalities in normal organs such as the lungs, liver, spleen, and kidneys in both groups (Fig. 7A, B). In the survival comparison from day 76 onwards in the control group where one death was confirmed, weight gain tended to increase and significantly increase on day 90 compared to the PD-L1-AAV particles treated group ($P<0.05$). Moreover, 5 (71.4%) of a total of 7 animals died on day 101. On the other hand, in the PD-L1-AAV particles treated group, 5 of a total of 8 (62.5%) were alive at day 101, and no significant weight loss associated with the use of PD-L1-AAV particles was observed during the observation period (Supplementary Fig. 5). In addition, there was no significant weight difference between the control-AAV particles treated group and the vehicle group of saline administration. Furthermore, to assess the side effects of PD-L1 AAV particles, immunohistochemical analysis was used to compare the expression of PD-L1 in each organ. Results showed no significant difference in PD-L1-positive cells in lungs, liver, spleen, and kidneys between the two groups (Fig. 8).

Discussion

Recently, the use of antibodies and inhibitor therapies that target molecules responsible for inducing immune tolerance in solid tumors has increased. However, the efficacy of these therapies has proved insufficient. Additionally, serious irAEs have been reported to occur. Therefore, developing novel inhibitory methods targeting tumor immune-tolerant molecules aiming to reduce adverse events and enhance efficacy is needed. Our previous study using a mouse ovarian cancer peritoneal dissemination model showed that tumor growth was significantly suppressed more potently in the PD-L1-KO ID8-inoculated group, with the complete absence of PD-L1 expression, than in the control ID8-inoculated group treated with anti-PD-L1 antibody therapy [9]. However, the technology of knocking out PD-L1 expression in tumor cells directly and efficiently in an in vivo model has not yet been established. Therefore, we focused on AAV-CRISPR/Cas9 and developed a mouse

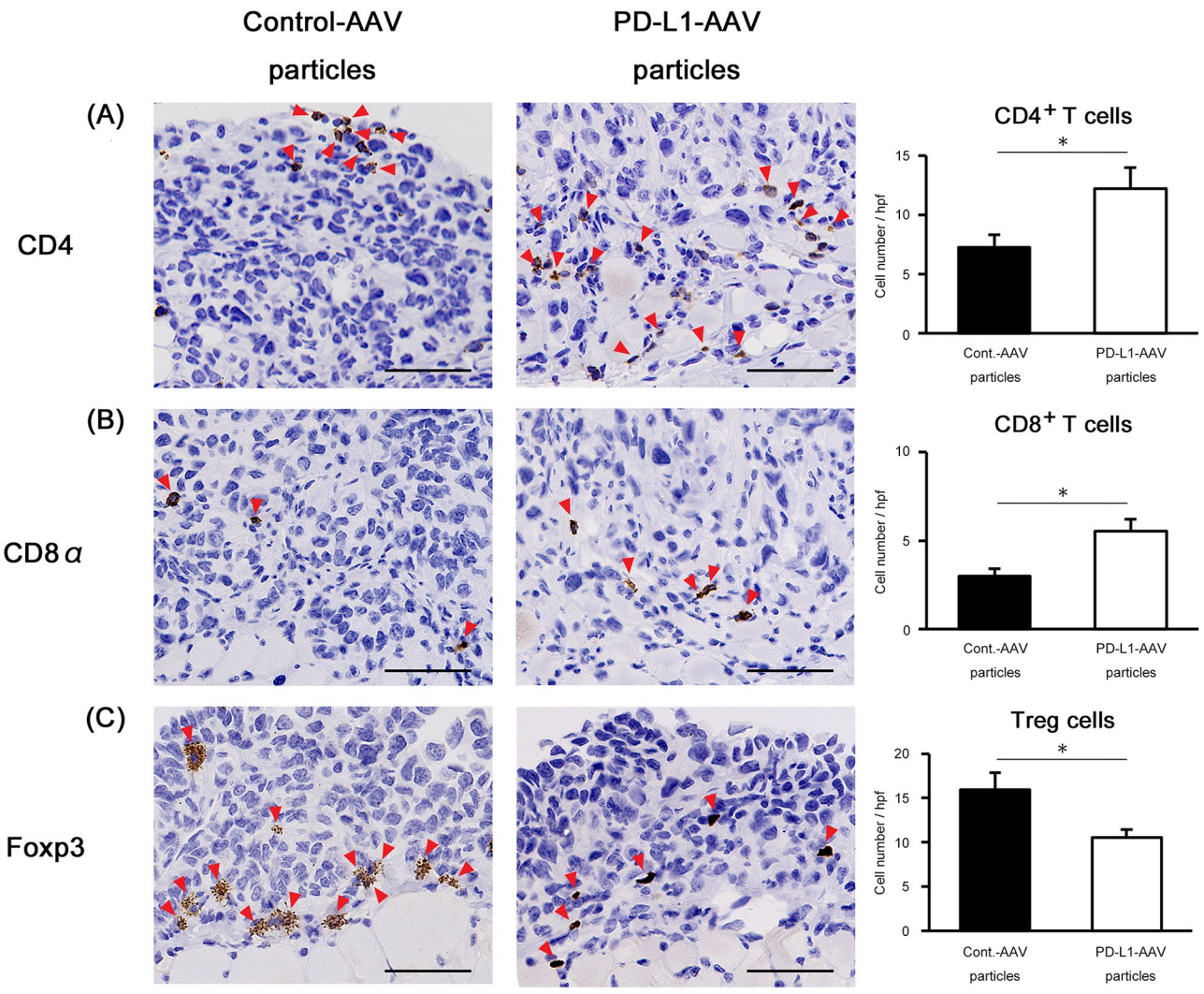

Fig. 5 Effect of multiple doses (9 times/week) of PD-L1 AAV particles on intratumoral lymphocyte infiltration in a mouse peritoneal dissemination model. (A–C) $CD4^+$ T, $CD8^+$ T, and Treg cells (Foxp3) in the tumor microenvironment of mice injected with PD-L1-AAV and control AAV particles on day 74. Representative results from eight independent experiments are shown. Red arrowheads indicate positive cells. Original magnification, ×400. Bar = 50 μm. The number of $CD4^+$ T, $CD8^+$ T, and Treg cells (Foxp3) in peritoneally disseminated tumors. All values are presented as mean ± SE ($n = 8$ in each group). Statistical significance was determined using an unpaired Student's t-test. * $p < 0.05$

model to directly disrupt PD-L1 expression in ovarian cancer in vivo. Gene delivery vectors based on AAV have been used in cancer gene therapy due to their safety and efficacy [23, 24].

Recently, clinical trials of gene therapy using the AAV-CRISPR/Cas9 system have been conducted in patients with Leber congenital amaurosis type 10 (NCT03872479), an inherited retinal disorder [25, 26], and in patients with advanced stage of various cancers [27]. In this study, a novel gene immunotherapy model targeting PD-L1 in ovarian cancer using AAV-CRISPR/Cas9 was developed, and its efficacy was investigated. This study showed that PD-L1 expression at the cellular level was significantly decreased following treatment with PD-L1-AAV particles compared with control-AAV particles. In the peritoneal dissemination model, PD-L1-AAV particles promoted anti-tumor immunity by increasing tumor-infiltrating lymphocytes within the tumor microenvironment, thereby suppressing ovarian cancer progression compared with those with control AAV particles. To the best of our knowledge, this is the first study to show the therapeutic impact of a novel gene immunotherapy model targeting PD-L1 in ovarian cancer cells using CRISPR/Cas9 genome editing.

Although the MOI and treatment time in each experiment differ, previous in vitro experiments using AAV-CRISPR/Cas9 have shown knockout efficiency of 15.6–30.7%, 43–56.7% [13], or 77–87% [18]. Furthermore, although knockout efficiency by PD-L1-AAV particles was relatively low in this study, a dominant

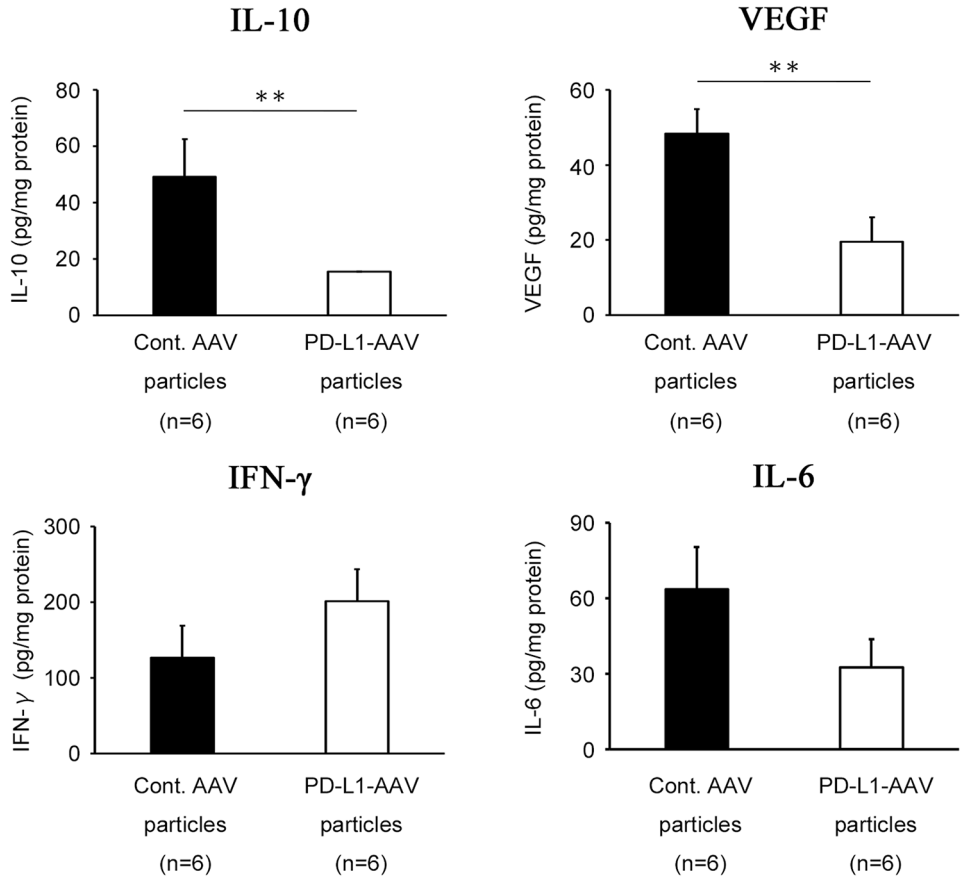

Fig. 6 Cytokine expression in disseminated tumors. Cytokine expression in disseminated tumors of mice injected with PD-L1-AAV and control AAV particles as measured by ELISA. All values represent the mean ± S.E. ($n = 6$). Statistical significance was determined using an unpaired Student's t-test; ** $P < 0.01$

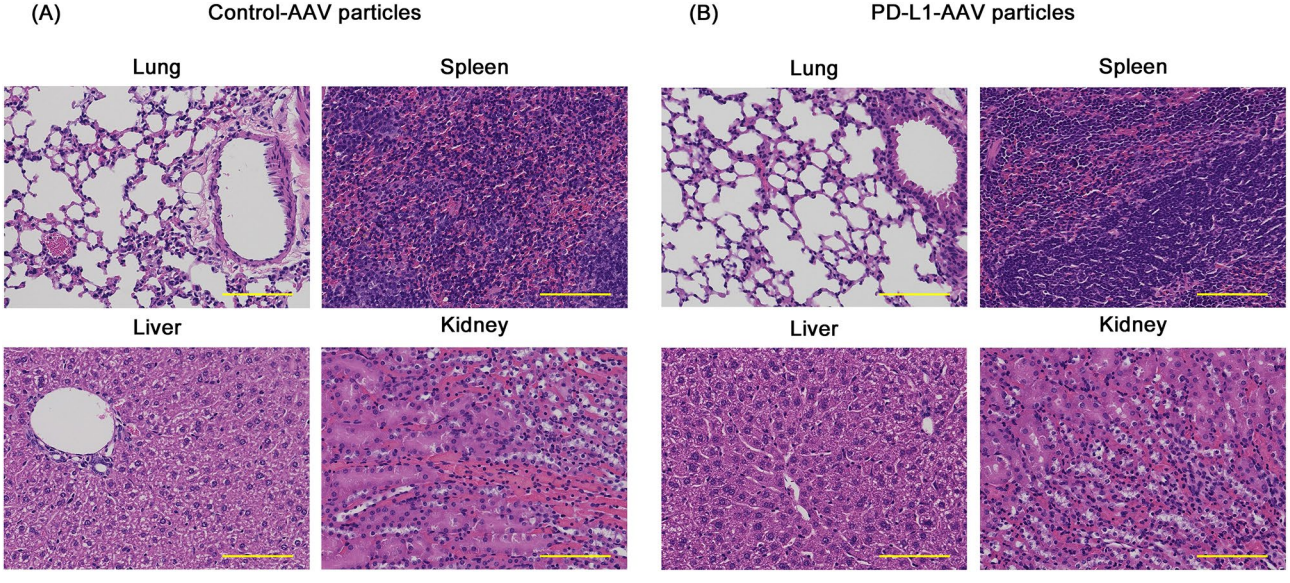

Fig. 7 The Adverse effects of PD-L1 AAV particles on normal organs in a mouse peritoneal dissemination model. Histopathological examination of normal organs, such as the lungs, liver, spleen, and kidneys, using hematoxylin and eosin staining 74 days after the injection of control AAV particles (**A**) and PD-L1-AAV particles (**B**). Original magnification, ×100. Bar = 100 μm

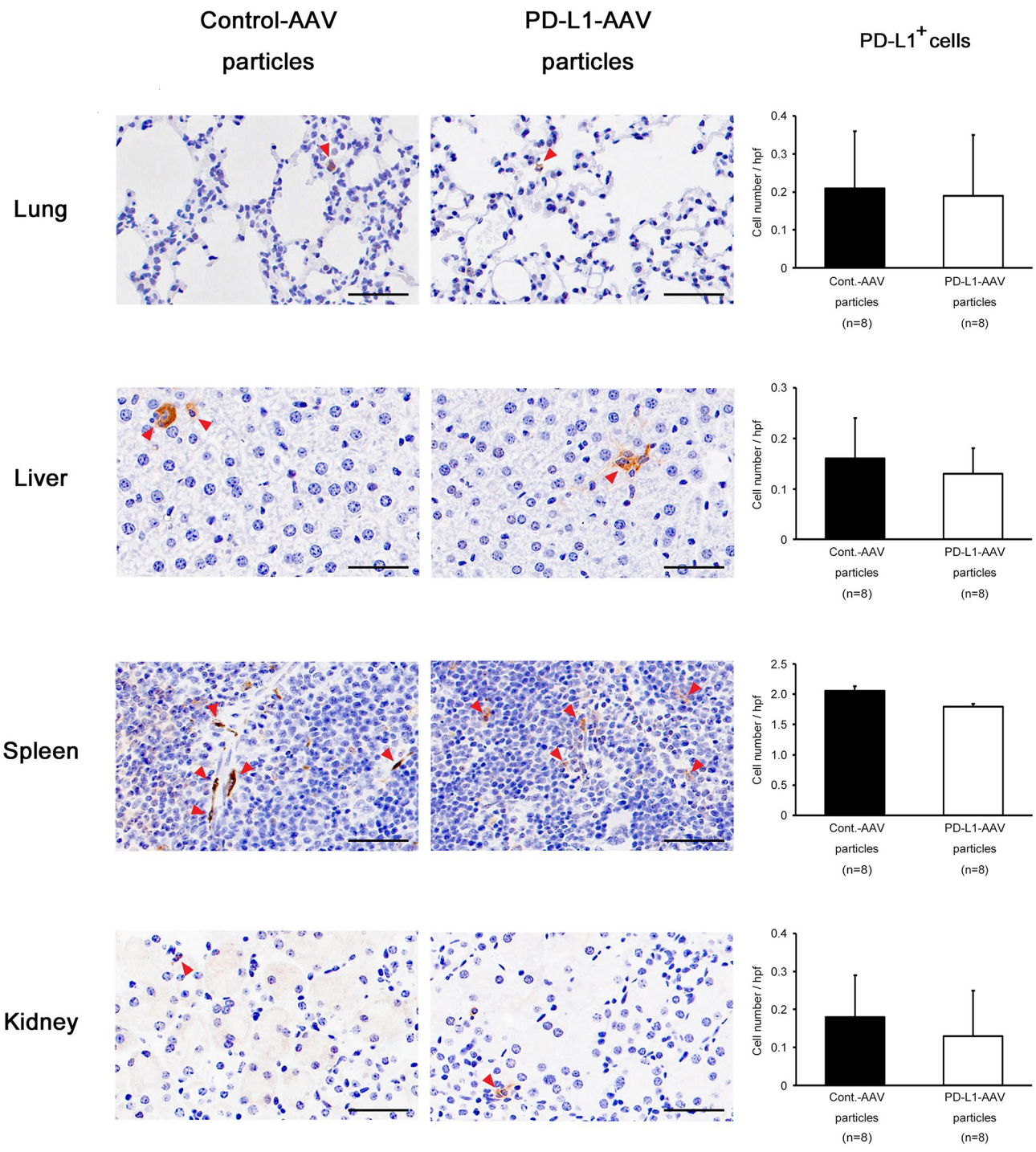

Fig. 8 Evaluation of PD-L1 expression in normal organs in a mouse peritoneal dissemination model. PD-L1 expression in normal organs, such as the lung, liver, spleen, and kidney of mice injected with PD-L1-AAV and control AAV particles on day 74 is shown. Red arrows indicate positive cells. Original magnification, x 400. Bar = 50 μm. The number of PD-L1-positive cells in normal organs. All values are presented as mean ± SE of 8 mice in both groups. Statistical significance was determined using unpaired Student's t-test

tumor suppressive effect was observed in vivo. In most previous studies [13, 18, 28], a single dose of AAV particles was used, with MOI being higher than the MOI used in this study. In this study, multiple doses (9 times, repeated every week) of PD-L1 AAV particles were used to compensate for the lower MOI values. Therefore, even at a low MOI of 35, multiple doses of AAV particles may result in a high knockdown efficiency of PD-L1 expression in vivo, which led to tumor suppression. Generally, the production of a large amount of viral vectors requires

a huge amount of labor and expense, so multiple doses of even a small amount of virus in this study could provide more clinically relevant treatment. On the other hand, it is possible that clinically, a higher MOI and less frequent dosing may result in better patient compliance, and in the future, this model may require the development of methods to purify large quantities of vectors to further increase the effectiveness of this approach and to reduce the frequency of injections as much as possible.

In this study, the combination of CRISPR/Cas9, an effective genome editing technique, and the AAV type 2 vector, which has high transduction efficiency for mouse ovarian cancer cells, showed high tumor-suppressing effects. In a survival comparison study, after day 76, when one death was observed in the control group, there was a trend toward weight gain in the control group compared to the PD-L1-AAV-treated mice, with significant weight gain on day 90, and 5 of a total of 8 mice (71.4%) died on day 101. On the other hand, 5 of a total of 8 (62.5%) PD-L1-AAV-treated mice were alive on day 101, and no significant weight loss associated with PD-L1-AAV particle administration was noted during the observation period. There was also no significant weight loss in the control-AAV particle treated group compared to the vehicle group of saline administration. To further evaluate the side effects of PD-L1 AAV particles, we also compared the expression of PD-L1 in each organ and found no significant difference in the number of PD-L1 positive cells in the lungs, liver, spleen, and kidneys in the two groups.

Recent studies have shown the tropism properties of AAV2 vectors for tumors [29–31]. AAV2 vectors showed higher transduction efficiency in hepatocellular carcinoma (HCC) than in normal hepatocytes [29], and c-Met played an important role in AAV2-mediated transduction in HCC [29–31]. C-Met is overexpressed not only in HCC but also in ovarian cancer [32]. In addition, c-Met is expressed in ovarian cancer cell line ID8, and activation of c-Met signaling promoted peritoneal dissemination of ovarian cancer [33]. Collectively, we speculate that PD-L1-AAV particles might exhibit higher transduction efficiency into tumor cells compared to normal organs due to its cancer tropism properties, leading to tumor suppression.

In advanced epithelial ovarian cancer, systemic treatment with intravenous chemotherapeutic agents for peritoneal metastases is considered less effective due to poor blood supply to the peritoneal surface and low drug penetration into the tumor [34]. Intraperitoneal chemotherapy can directly contact the intraperitoneal tumors and produce cytotoxic effects by convection and diffusion. Additionally, the peritoneal-plasma barrier blocks the leakage of the chemotherapeutic agent from the peritoneal cavity and tumor tissue into local vessels, thus maintaining a high chemotherapeutic agent concentration in the tumor tissue to promote tumor apoptosis and a low chemotherapeutic agent concentration in local blood vessels to reduce systemic toxicity in the molecular and cellular mechanisms [35]. Similarly, for the AAV particles used in this study, systemic toxicity may be reduced when used intraperitoneally compared with systemic treatment with intravenous. Thus, the study results suggest that intraperitoneal administration of AAV particles of the CRISPR/Cas9 system targeting PD-L1 on tumors is a safer treatment with less impact on normal organs and potentially fewer side effects than intravenous administration, which is systemic administration.

In our previous study [9], we used the CRISPR/Cas9 system to generate a stable ovarian cancer cell line exhibiting the deletion of the PD-L1 gene in mouse ovarian cancer cells (PD-L1-KO ID8). In an intraperitoneal injection mouse peritoneal dissemination model, we demonstrated that, compared to the control group, tumor-infiltrating lymphocytes significantly increased in the tumor microenvironment. Additionally, by regulating cytokines and chemokines at the mRNA level, we promoted anti-tumor immunity and suppressed tumor growth. Even in a previous study, Reyes RM et al. demonstrated that in a mouse model of bladder cancer with lung metastasis, no therapeutic effect was observed with anti-PD-L1 inhibitors or IL-2 drugs alone, but that their combination significantly improve survival compared to the control group. This showed that in addition to anti-PD-L1 inhibitors, IL 2 and CD8-positive T cells are activated thereby demonstrating tumor suppressive effects [36]. Furthermore, CXCL9 was shown to be one of the genes that contribute to prolonged prognosis along with IFN-γ in cases of metastatic malignant melanoma successfully treated with anti-PD-1 inhibitors. Moreover, CXCL9 produced by dendritic cells and macrophages activated by IFN-γ produced by T cells and NK cells was shown to induce CD8-positive T cells and suppress tumor growth [37]. The above suggests that cytokines and chemokines also contribute to anti-tumor immunity. Similarly, in the present study, we demonstrated that the number of intratumoral $CD4^+$ and $CD8^+$ T cells was significantly higher, whereas that of $Foxp3^+$ Treg cells was significantly lower in the PD-L1-AAV particles treated group than in the control group. In addition, there was a significant decrease in protein expression of immunosuppressive cytokines IL-10 and VEGF in PD-L1-AAV particles treated groups. We speculate that suppression of PD-L1 in tumors by PD-L1-AAV particles may promote anti-tumor immunity via increasing tumor-infiltrating lymphocytes as well as modulating cytokine production and suppress ovarian cancer progression. Future studies will increase the knockout efficiency of PD-L1-AAV particles and further elucidate the mechanism at the molecular level.

Recent studies have revealed that PD-L1 is secreted in the form of exosomes and/or soluble proteins and that exosomal PD-L1 has substantial biological activity at the extracellular level [38]. These proteins suppress T cell immunity and are associated with poor prognostic factors in patients with head and neck squamous cell carcinoma [39] or lung cancer [40]. In addition, the expression of PD-L1 is intricately regulated by several processes, such as gene transcription, post-transcriptional and post-translational modifications, and these pathways increase PD-L1 expression and enhance cancer immune escape [38]. Therefore, we will demonstrate how AAV-CRISPR/Cas9 therapy targeting PD-L1 is involved in PD-L1 regulatory mechanisms at the transcriptional, post-transcriptional, post-translational modifications, and extracellular levels. We believe that this therapy can provide significant benefits for patients with ovarian cancer patients.

In conclusion, the study results demonstrated for the first time that disrupting the PD-L1 gene expression using the AAV-CRISPR/Cas9 system can inhibit cellular proliferation and promote anti-tumor immunity by increasing tumor-infiltrating lymphocytes within the tumor microenvironment in ovarian cancer. Therefore, targeting the PD-L1 gene using the AAV-CRISPR/Cas9 system may be a new therapeutic approach for ovarian cancer.

Abbreviations

PD	L1-programmed death ligand 1
AAV	CRISPR/Cas9-adeno-associated virus-clustered regularly interspaced short palindromic repeats/Cas9

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-14093-0.

Supplementary Material 1: Full scan image of the western blot analysis used in the manuscript for Fig. 2A

Supplementary Material 2: The multiple exposure images before processing captured under different conditions of the western blot analysis used in the manuscript for Fig. 2A.

Supplementary Material 3: Full scan image of the western blot analysis used in the manuscript for Fig. 4A.

Supplementary Material 4: Cytokine and chemokine expression in disseminated tumors. Cytokine and chemokine expression in disseminated tumors of mice injected with PD-L1-AAV and control AAV particles as measured by real-time RT-PCR. All values represent the mean ± S.E. (n=6). Statistical significance was determined using an unpaired Student's t-test.

Supplementary Material 5: Body weight of mice treated with PD-L1-AAV particles (n=8), mice treated with control-AAV particles (n=8), and mice treated with saline (n=2) in a survival comparison. All values represent the mean ± S.E.. Statistical significance was determined using an unpaired Student's t-test. * $p < 0.05$.

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Author contributions

T.Y., S.T., and K.I. designed the methodology and wrote the manuscript. T.Y., S.T., I.S., N.I., M.F., K.N., T.N., and Y.T-H. participated to perform the cell culture, construction of the plasmid vector, western blot analysis, immunohistochemical analysis, real time RT-PCR, ELISA and in vivo experiments. T.Y., S.T., and K.I. analyzed and interpreted the data. T.Y., S.T., K.I., and K.I. confirm the authenticity of all raw data. All authors read and approved the final version of the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Institutional Review Board Statement: All animal care and animal experimental procedures were performed in accordance with the Committee on Animal Care and Use of Wakayama Medical University (approval number 964) and all experiments with recombinant DNA technology were approved by the DNA Recombination Experiment Committee of Wakayama Medical University (approval numbers 29–34 and 2021-71).

Consent for publication

Not applicable.

Clinical trial number

Not applicable.

Competing interests

The authors declare no competing interests.

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