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Anti-PD-1 antibody combined with chemotherapy suppresses the growth of mesothelioma by reducing myeloid-derived suppressor cells

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ABSTRACT

Background: The combination of anti-PD-1/PD-L1 antibody with chemotherapy has been approved for the first-line therapy of lung cancer. However, the effects against malignant mesothelioma (MPM) and the immunological mechanisms by which chemotherapy enhances the effect of targeting PD-1/PD-L1 in MPM are poorly understood.

Materials and Methods: We utilized syngeneic mouse models of MPM and lung cancer and assessed the therapeutic effects of anti-PD-1 antibody and its combination with cisplatin (CDDP) and pemetrexed (PEM). An immunological analysis of tumor-infiltrating cells was performed with immunohistochemistry.

Results: We observed significant therapeutic effects of anti-PD-1 antibody against MPM. Although the effect was associated with $CD8^+$ and $CD4^+$ T cells in tumors, the number of $Foxp3^+$ cells was not reduced but rather increased. Consequently, combination with CDDP/PEM significantly enhanced the antitumor effects of anti-PD-1 antibody by decreasing numbers of intratumoral myeloid-derived suppressor cells (MDSCs) and vessels probably through suppression of VEGF expression by CDDP + PEM.

Conclusions: The combination of anti-PD-1 antibody with CDDP + PEM may be a promising therapy for MPM *via* inhibiting the accumulation of MDSCs and vessels in tumors.

1. Introduction

Malignant pleural mesothelioma (MPM) is a rare and aggressive malignant tumor primarily affecting the pleura with approximately 80 % of cases resulting from occupational or environmental asbestos exposure [1–4]. The MPM incidence is suspected to increase in the future in many countries in which asbestos either has not yet been banned or is still largely used. The prognosis of MPM is very poor, with a median survival time between 12 and 18 months and a 5-year survival rate of < 5%, irrespective of multimodal therapies [1–4]. The high mortality rates of MPM may be primarily due to the difficulty of making an early diagnosis and the poor responses to chemotherapy or radiotherapy. In addition, although the first-line treatment for patients with MPM who are not eligible for surgery is established as platinum-based chemotherapy with pemetrexed (PEM), no approved second-line therapy has been developed yet except nivolumab in Japan [1–4].

Recent advances in cancer immunotherapy have resulted in the development of immune checkpoint inhibitors (ICIs) of several antibodies for cytotoxic T-lymphocyte antigen protein 4 (CTLA4), programmed cell death 1 (PD-1) and its ligand PD-L1, which have been approved for various malignancies, including melanoma, lung cancer, renal cell cancer, head and neck cancer, Hodgkin's disease and urothelial cancer [5,6]. The efficacy of ICIs for patients with advanced MPM has been also demonstrated in several studies [7–10]. In addition, clinical trials involving combination therapy of anti-PD-1/PD-L1 antibodies with other modality have demonstrated the promising efficacy of this regimen against various cancers [11–16].

Among those combined therapies, the immunological mechanisms underlying the combination of anti-PD-1 antibody with anti-CTLA4 antibody has previously been reported [17–20]. Das et al. detected the drastic upregulation of many genes related to the activation and proliferation of human T cells harvested from peripheral blood of patients

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treated with both anti-PD-1 and CTLA-4 antibodies compared to either monotherapy [17]. In murine tumor models, the activation of CD8 + and effector CD4 + T cells has been shown to be associated with the reduced number of regulatory T (Treg) cells in tumors [18,18,19,20].

However, the mechanisms involved in combined therapy with anti-PD-1/PD-L1 antibody and chemotherapy have not yet been fully clarified in MPM or other tumors. We therefore assessed the combined effect of anti-PD-1 antibody and cisplatin (CDDP) + PEM in MPM model mice. We also investigated the cellular and molecular mechanisms underlying the effects of this therapy.

2. Materials and methods

2.1. Cell lines

The mouse MPM cell line AB1-HA, a transfectant with the gene encoding influenza HA into AB1 cells, were purchased from Public Health England (London, UK). The mouse lung cancer cell line Lewis lung carcinoma (LLC) was purchased from ATCC (Manassas, VA, USA). These cell lines were maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (50 μg/mL). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Reagents

Recombinant murine interferon (IFN)-γ was purchased from PEPROTECH (Rocky Hill, NJ, USA). CDDP and PEM were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Animals

Six-week-old male C57BL/6 J mice and BALB/c mice were obtained from Charles River Japan Inc. (Shiga, Japan). Mice were maintained under specific-pathogen-free conditions throughout this study. All experiments were performed in accordance with the guidelines established by Tokushima University Committee on Animal Care and Use. At the end of each *in vivo* experiment, the mice were euthanized by cervical dislocation. All experimental protocols were reviewed and approved by the animal research committee of Tokushima University, Japan.

2.4. Flow cytometry

The PD-L1 or PD-L2 expression of AB1-HA or LLC cells cultured with or without 10 ng/mL IFN-γ, 0.5 μM CDDP or 0.01 μM PEM for 24 h was detected by flow cytometry using BD FACSDiva™ (BD Bioscience, San Diego, CA, USA). PE-conjugated anti-mouse PD-L1 antibody and anti-mouse PD-L2 antibody were purchased from eBioscience (San Diego, CA, USA).

2.5. Cell proliferation assays

The *in vitro* proliferation of cells was assessed *via* the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction method as reported previously [21]. In brief, tumor cells seeded in a 96-well plate were cultured with anti-mouse PD-1 antibody or chemotherapeutic agents. After 48 − 72 h, MTT was added to each well, and the absorbance was measured with a SUNRISE Remote R microplate reader (Tecan, Mannedorf, Switzerland) at test and reference wavelengths of 550 and 630 nm, respectively.

2.6. In vivo mouse model

AB1-HA cells (1×10^6 cells/mice) and LLC cells (5×10^5 cells/mice) were subcutaneously injected into C57BL/6 and BALB/c mice, respectively. Once the tumor reached a diameter of 4 − 5 mm, anti-PD-1 antibody (100 μg/mouse; ONO Pharmaceutical Co., Ltd., Osaka, Japan) was administered intraperitoneally every 3 days for a total of 3 doses. For the combination with the chemotherapeutic agents, CDDP (3 μg/g) on day 1 and PEM (30 μg/g) on days 1–5 were administered intraperitoneally from the start of the treatment. The tumor size was measured using vernier calipers at least twice a week using the following formula: Volume = $ab^2 2^{-1}$ (a, long diameter; b, short diameter). When the average tumor size reached 2000 − 3000 mm^3, the mice were killed humanely, and the tumors were resected for further analyses. To deplete myeloid-derived suppressor cells (MDSCs), 100 μg of anti-mouse Ly6G/Ly6C (Gr-1) antibody (Clone: RB6 − 8C5) (BioXCell, New Hampshire, NH, USA) was injected intraperitoneally every 3 days.

2.7. Immunohistochemistry

Mouse subcutaneous tumors were fixed in 10 % formalin for 24 h and embedded in paraffin, or in optimal cutting temperature (OCT) compound, after which they were stored at − 80 °C. The tumor sections (4-8 μm thick) were incubated with rabbit anti-PD-L1 polyclonal antibody (1:200 dilution; Proteintech, Tokyo, Japan), rabbit anti-CD4 monoclonal antibody (1:100 dilution; Abcam, Cambridge, MA, USA), rabbit anti-CD8 polyclonal antibody (1:200 dilution; Bioss, Woburn, MA, USA), rat anti-Foxp3 antibody (1:200 dilution; eBioscience), rat anti-Ly6G (Gr1) antibody (1:100 dilution; eBioscience), rabbit anti-Ki67 monoclonal antibody (1:200 dilution; Abcam), rat anti-CD31 antibody (1:150 dilution; BD Pharmingen, Franklin Lakes, NJ, USA) or rabbit anti-VEGFA antibody (1:400 dilution; Abcam) overnight at 4 °C. These sections were re-incubated with appropriate secondary antibodies and conjugated with peroxidase (Nichirei, Tokyo, Japan). Immunoreactivity was detected by using the DAB Liquid System (Nichirei, Tokyo, Japan), and samples were counterstained with hematoxylin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining (TAKARA BIO, Shiga, Japan) was also performed according to the manufacturer's protocol in order to detect apoptotic cell death in the tumor sections. The number of positive cells was counted at a × 400 magnification. Images were acquired by microscopy (BZ-9000; Keyence, Tokyo, Japan). The data were expressed as % positive cell ratio (the number of staining-positive cells/the number of total cells) from randomly selected multiple fields. For immunofluorescence staining, purified rat anti-mouse CD8a (clone 53 − 6.7), (1/150 dilution, BD bioscience), purified rat anti-mouse CD4 (Clone H129.19) (1/150 dilution, BD pharmingen), anti-VEGF-A antibody, ab46154 polyclonal (1/50 dilution, abcam) and anti-interferon gamma antibody, rabbit polyclonal (1/50 dilution, abcam) were used as primary antibodies, and goat anti-rat IgG (H + L) cross-adsorbed antibody, Alexa Fluor 594 (1/250 dilution, Thermo) and goat anti-rabbit IgG (H + L) cross-adsorbed antibody, Alexa Fluor 488 (1/250 dilution, Thermo) were used as secondary antibodies, respectively. Nuclei were counterstained with DAPI (blue). For measurement of stained area for VEGF, images were acquired by Keyence BZ-9000 microscopy (Keyence, Tokyo, Japan) and quantified with an ImageJ software to show the number of pixels over appropriate threshold.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The mouse VEGF protein level in the culture supernatant of tumor cells was assessed with a Quantikine Mouse VEGF Imuunoassay (R&D system, Minneapolis, MN, USA) according to the manufacturer's protocol. The culture supernatant was collected after exposure to 0.25 μM CDDP or 0.005 μM PEM for 48 h.

2.9. Western blot analysis

Tumor tissues were homogenized in M-PER reagents (Thermo)

containing phosphatase and protease inhibitor cocktails (Roche, Basel, Switzerland) [21]. Protein concentrations were measured using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA, USA). The same amounts (5 μg) of extract proteins were used for the Simple Western™ System (ProteinSimple, Santa Clara, CA, USA). We used the Simple Western™ System as described in a previous report [22] and according to the manufacturer's instructions, and we analyzed the protein amounts based on the signal intensity.

2.10. Statistical analyses

The data are presented as the means ± standard error of the mean. The statistical analyses were performed using Student's *t*-test for unpaired samples. A one-way analysis of variance followed by Tukey's multiple-comparison post-hoc test was also used as appropriate. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effects of anti-PD-1 antibody monotherapy on mouse MPM or lung cancer cells

As the first set of experiments, we evaluated whether or not the blockade of the PD-1/PD-L1 axis inhibited the growth of murine tumors, MPM (AB1-HA) and Lung cancer (LLC) *in vivo*. Mice bearing subcutaneous tumors with AB1-HA or LLC cells were treated with anti-PD-1 antibody. A total of three doses of anti-PD-1 treatment subsequently inhibited the tumor growth in both tumor models (Fig. 1A). In order to assess the mechanism of action of anti-PD-1 antibody, we immunohistochemically confirmed the expression of PD-L1 in the subcutaneous tumor. As shown in Fig. 1B, both tumor cells expressed PD-L1 *in vivo*; however, these tumor cells were found to be negative for PD-L1 expression *in vitro*, and its expression was enhanced when cells were treated with IFN-γ (Fig. 1C), suggesting that the up-regulation of cytokines/chemokines, including IFN-γ, by the cross-talk between tumor cells and host cells regulated the expression of PD-L1 in tumor cells *in vivo*. In fact, IFN-γ was detected in both $CD4^+$ and $CD8^+$ T cells infiltrating into AB1-HA tumors (Supplementary Fig. 1). In contrast, the expression of PD-L2 was negative regardless of IFN-γ treatment in both cell lines. We also confirmed by the MTT assay that the anti-PD-1 antibody itself did not inhibit the proliferation of tumor cells, while cytotoxic drugs such as CDDP or PEM strongly inhibited the tumor-cell proliferation in a dose-dependent manner (Fig. 1D).

3.2. Recruitment of host immune cells by anti-PD-1 antibody treatment

Since the recruitment of host immune cells is critical for the antitumor action of immunotherapy, we assessed a series of immune cells that had infiltrated the tumor tissue. An immunohistochemical analysis revealed that the numbers of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells were significantly increased following anti-PD-1 antibody treatment in LLC (Fig. 2A) and AB1-HA (Fig. 2B). In terms of immunosuppressive cells, the number of $Foxp3^+$ regulatory T cells (Tregs) was also increased by anti-PD-1 antibody treatment, whereas the number of $Gr1^+$ myeloid-derived suppressor cells (MDSCs) did not change. These data suggested that treatment with anti-PD-1 antibody enhanced the migratory and invasive ability of cytotoxic T lymphocytes (CTLs) and effector $CD4^+$ T cells, which may have helped CTLs kill tumor cells, although these effects may have been limited by the increase in the number of Tregs.

3.3. Effects of combination therapy of chemotherapy and anti-PD-1 antibody on the growth of MPM

We next examined whether or not the combination of cytotoxic chemotherapeutic agents with anti-PD-1-antibody could further inhibit the tumor growth *in vivo*. After the subcutaneous injection of AB1-HA cells, tumor-bearing mice were treated with CDDP + PEM and/or anti-PD-1 antibody (Fig. 3A). As shown in Fig. 3B, treatment with chemotherapeutic agents or anti-PD-1 antibody inhibited the tumor growth to some extent compared to the control group, whereas combination therapy significantly suppressed tumor growth compared to single treatment with each agent alone. Similarly, combined therapy enhanced antitumor effects as compared to each monotherapy against LLC.

Regarding the mechanism underlying the effect of the combination therapy, we first hypothesized that the chemotherapeutic agents modified the expression of PD-L1 on tumor cells. As shown in Fig. 3C, neither CDDP nor PEM modified the expression of PD-L1 on AB1-HA cells *in vitro*. An immunohistochemical analysis showed no marked difference in the expression of PD-L1 in the subcutaneous tumor among the groups (Fig. 3D).

3.4. Changes to immune cell recruitment in tumors treated with combination therapy against MPM

When tumor-infiltrating immune cells were assessed by immunohistochemistry, we observed a significant increase in the number of $CD4^+$ T cells in the tumors treated with combination therapy (Fig. 4), whereas the numbers of $CD8^+$ T cells and $Foxp3^+$ Tregs did not change, regardless of the addition of chemotherapy to anti-PD-1 antibody monotherapy. Of note, the number of $Gr1^+$ MDSCs was significantly reduced when tumors were treated with CDDP + PEM. These results suggested that, in addition to its cytotoxic mechanism, CDDP or PEM has the ability to prevent MDSCs from infiltrating the tumor, which may help CTLs kill as many tumor cells as possible. This would explain how combination therapy exhibited particularly strong inhibition of tumor growth. This hypothesis was supported by our assessment of tumor cell death using TUNEL staining showing that combination therapy significantly enhanced tumor cell death compared to individual treatment (Fig. 4). However, by contrast, Ki67 staining showed that the proliferation of tumor cells was not changed when anti-PD-1 antibody was added to CDDP + PEM.

Reduction of MDSCs contributes to enhanced antitumor effects by anti-PD-1 antibody through inhibition of tumor vessel formation by chemotherapy-mediated suppression of VEGF expression

VEGF was previously reported to play a key role in the differentiation and accumulation of MDSCs [14–16]. We therefore examined whether or not CDDP or PEM affected the VEGF expression in tumor cells. In our *in vitro* analysis, CDDP significantly reduced the expression of VEGF protein in AB1-HA cells at a concentration that did not affect the tumor cell growth (Fig. 5A and B). PEM also inhibited the VEGF protein expression, although its effect was weaker than that of CDDP. Furthermore, the VEGF expression in the subcutaneous tumor formed by AB1-HA cells was decreased when mice were treated with CDDP + PEM (Fig. 5D). These data collectively suggested that the suppression of the VEGF expression in tumors by chemotherapeutic agents, such as CDDP and/or PEM, might be a mechanism underlying the reduced accumulation of MDSCs in addition to anti-angiogenic effects (Fig. 5E) in the tumor. The hypothesis was confirmed by depleting MDSCs with anti-Gr-1 antibody. Treatment with anti-Gr-1 antibody enhanced antitumor effects of anti-PD-1 antibody, suggesting that the depletion of MDSCs is a promising strategy to enhance antitumor immunity mediated by anti-PD-1 antibody (Fig. 6).

4. Discussion

In the present study, we found that combination immunotherapy with anti-PD-1 antibody and chemotherapy effectively inhibited the growth of malignant mesothelioma AB1-HA in mice. The additional effects of CDDP/PEM to anti-PD-1 therapy appear to have been mediated by reducing the number of intratumoral MDSCs as a result of

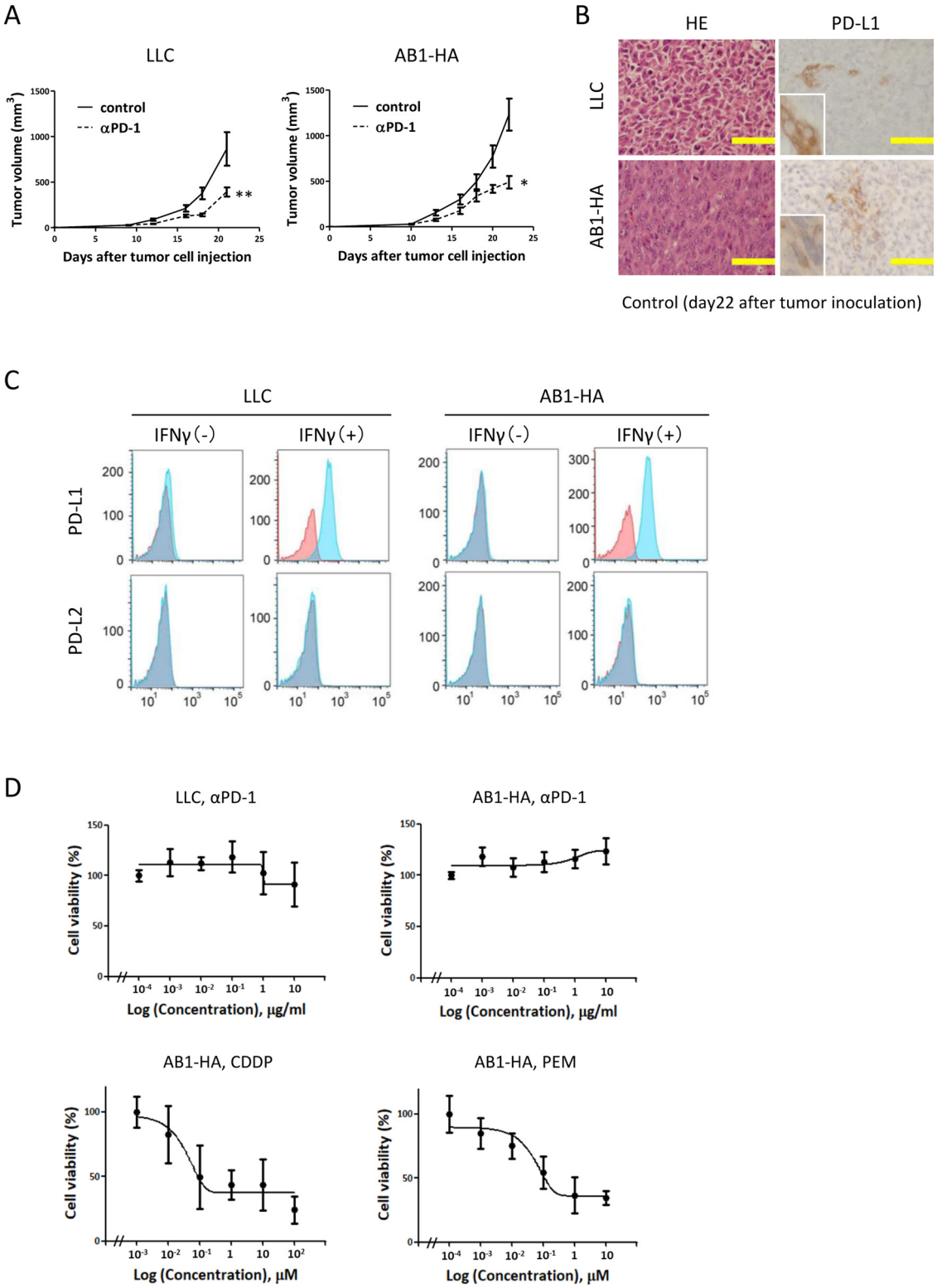

Fig. 1. Anti-tumor effects of anti-PD-1 antibody and the expression of PD-L1 on MPM and lung cancer cells. (A) The effect of anti-PD-1 antibody of mouse subcutaneous tumor. $*P < 0.01$, $**P < 0.05$ compared to the control group. (B) The immunohistochemical analysis of PD-L1 in mouse subcutaneous tumors. LLC and AB1-HA tumors in control groups were harvested on day 22 and then stained with HE and anti-PD-L1 antibody. Scale bar, 500 μm. Magnification, ×400, x1600 (inside window). (C) A flow cytometric analysis of the PD-L1 or PD-L2 expression on tumor cells with or without IFN-γ treatment (10 ng/mL for 24 h) *in vitro*. (D) The effect of the anti-PD-1 antibody, CDDP or PEM on tumor cell proliferation examined by an MTT assay. Data are shown as the mean ± SEM.

decreasing the production of VEGF in tumors.

Combined immunotherapy with anti-PD-1/PD-L1 antibody and chemotherapy is approved only for non-small cell lung cancer (NSCLC). The tumor proportion score (TPS) of the PD-L1 expression has been reported to be associated with the tumor response to treatment with anti-PD-1 antibody in NSCLC as well as MPM in most, but not all cases [7,9,10,15,16,23]. Although the rate of patients with NSCLC showing ≥1% and ≥50 % of TPS of PD-L1 expression have been reported to be

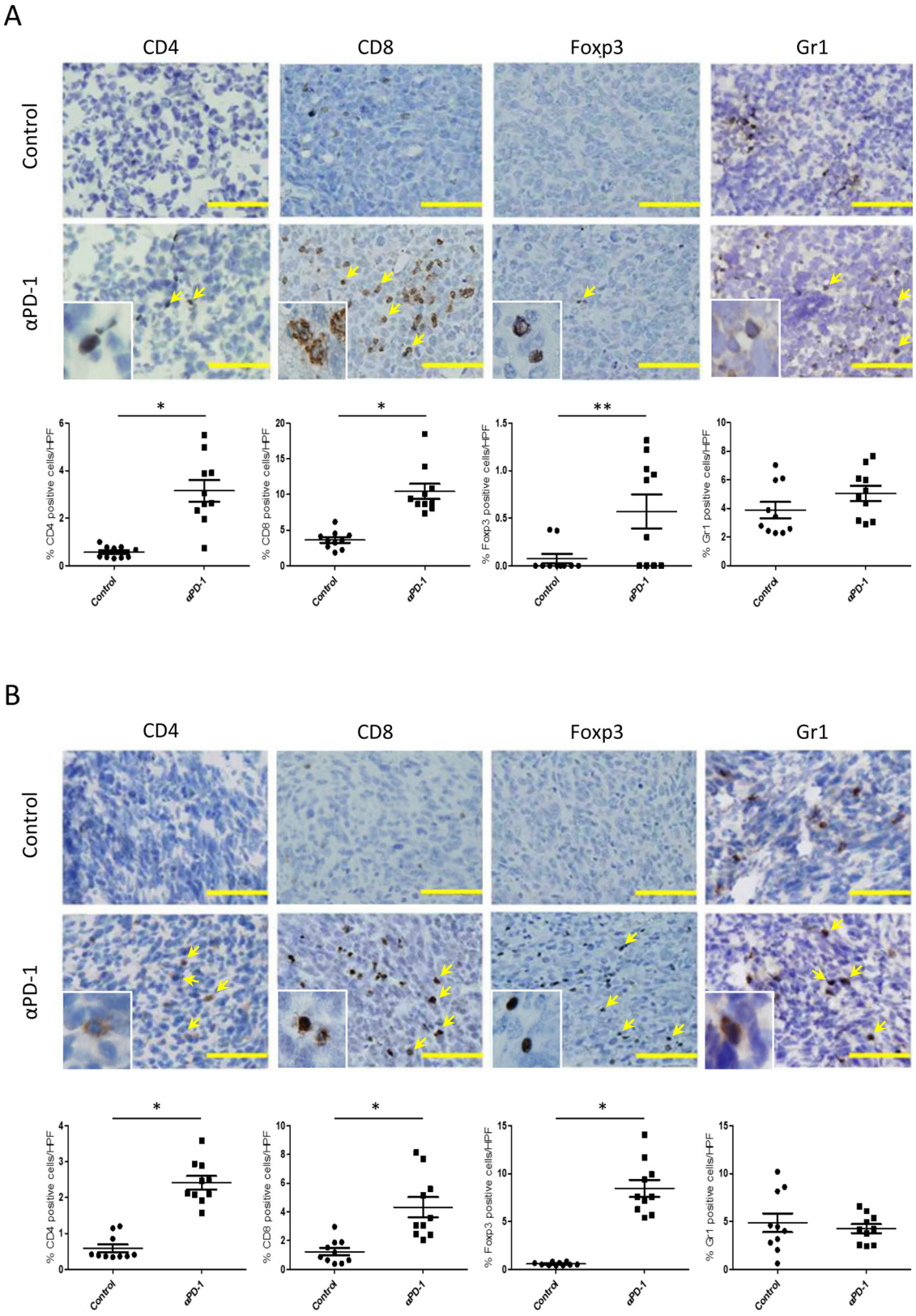

Fig. 2. Immunohistochemical analysis of tumor-infiltrating CD4-, CD8-, Foxp3- and Gr1-positive cells in mouse subcutaneous tumors comprising (A) LLC cells or (B) AB1-HA cells. Infiltration of immune cells into tumors (day 22) was analyzed with an immunostaining. The stained cells were shown to be brown. CD4, CD8 and Gr-1 localized to cell membrane, and Foxp3 localized to nucleoplasm were detected in immune cells infiltrating into tumors. The arrows in the pictures indicate the positive cells for PD-L1 staining. Scale bar, 500 μm. Magnification, × 400, x1600 (inside window). *$P < 0.001$, **$P < 0.01$. Data are shown as the mean ± SEM.

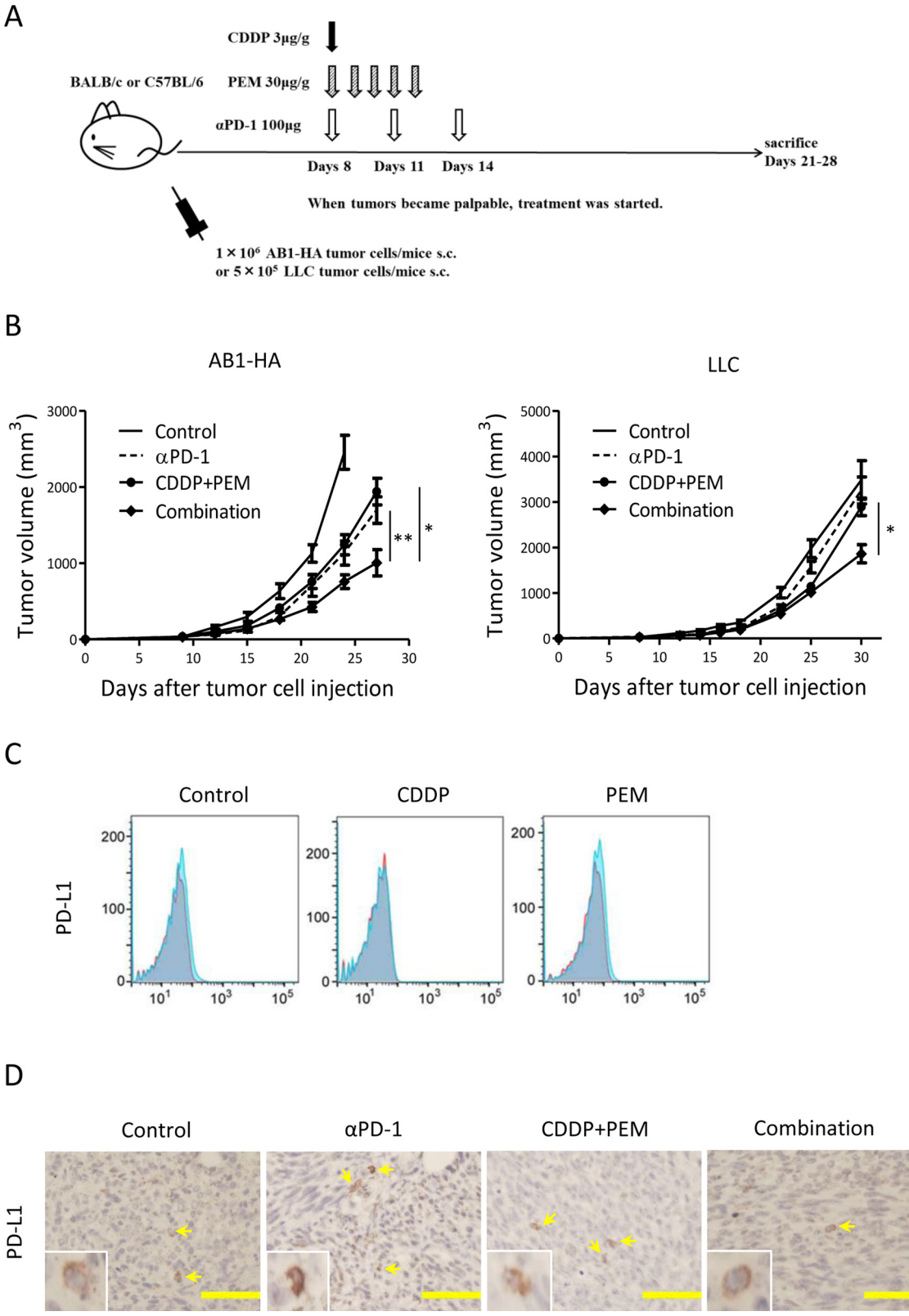

Fig. 3. Therapeutic efficacy of anti-PD-1 antibody combined with chemotherapeutic agents against AB1-HA tumor. (A) The treatment schedule for the combination therapy. (B) The effects of the treatment (shown as the tumor volume). $*P < 0.01$, $**P < 0.05$. Data are shown as the mean ± SEM. (C) Results of a flow cytometric analysis of PD-L1 on AB1-HA cells that were treated with CDDP (0.5 μM) or PEM (0.01 μM) for 24 h *in vitro*. (D) Results of an immunohistochemical analysis of PD-L1 in each group of mouse subcutaneous tumor. PD-L1 was stained on cell membrane of some of tumor cells. Scale bar, 500 μm. Magnification, × 400, x1600 (inside window).

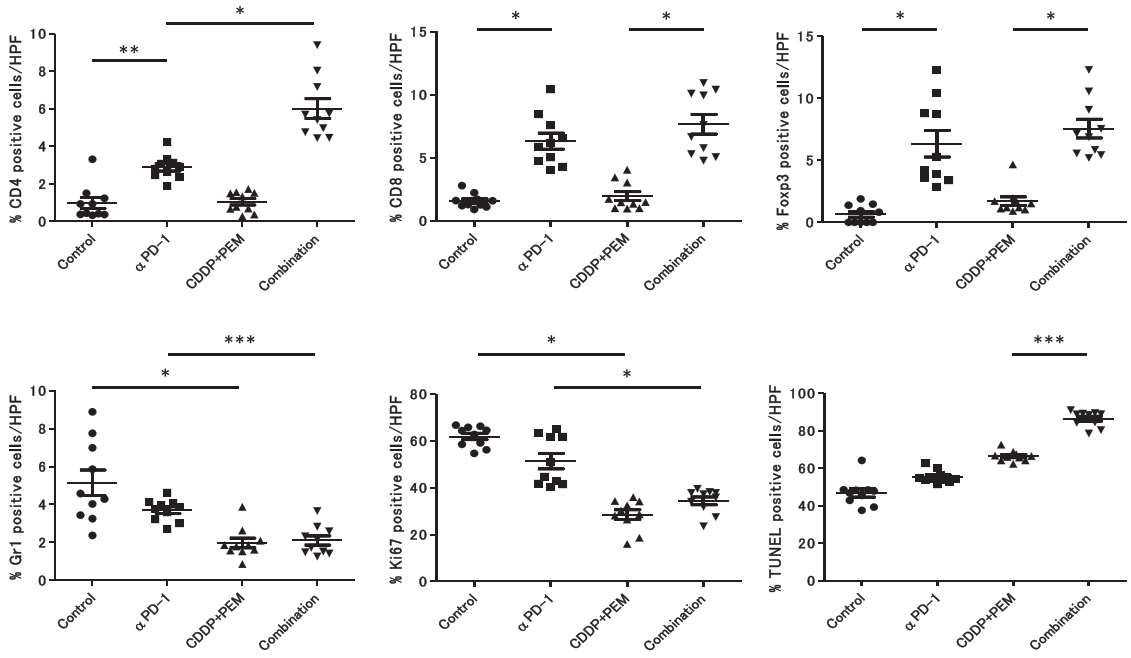

Fig. 4. Immunohistochemical analyses of subcutaneous AB1-HA tumor treated with anti-PD-1 antibody, CDDP + PEM or their combination. Antibodies for CD4, CD8, Foxp3, Gr-1 and Ki67 were used to detect the positive cells by immunostaining (see Supplementary Fig. 2). The stained cells were detected as described in the legend of Fig. 2. In TUNEL staining, the positive cells showing brown nuclei were counted. The data were expressed as % positive cell ratio (the number of staining-positive cells/the number of total cells). Data are shown as mean ± SEM. $*P < 0.001$, $**P < 0.01$, $***P < 0.05$.

high at 60 %–70 % and 25 %–35 %, respectively [15,16,23], those rates in MPM are only 35 %–60% and 5 %–15 %, respectively [7,9,10], indicating that combined immunotherapy with anti-PD-1/PD-L1 antibody and other modalities is required for MPM to enhance the clinical benefits of therapy.

Chemotherapy is expected to be an effective modality in combination with ICIs because cytotoxic drugs have the ability to stimulate tumor immunity by affecting both cancer cells and immune cells [24,25]. Immunogenic cell death (ICD) of tumor cells may stimulate the antigen-presenting pathway by releasing immuno-stimulatory molecules, such as calreticulin, annexin A1, ATP and high-mobility group box 1. However, ICD seems to be difficult to evaluate *in vivo*, so substantial focus has been paid to immunosuppressive cells, including Treg cells and MDSCs, which are decreased by treatment with anti-cancer drugs. Previous reports showed that cyclophosphamide (CPA) and gemcitabine (GEM) clearly reduced the number of Treg cells in mice and humans [26–29]. Furthermore, 5-fluorouracil (5-FU), GEM, cisplatin (CDDP) and docetaxel (DOC) were shown to decrease MDSCs [29–32]. However, the effects of pemetrexed (PEM), a key drug for MPM as well as nonsquamous NSCLC, and the combination regimen of PEM + CDDP on Treg and MDSCs has not yet been evaluated. In addition, although there have been a few reports describing the combined antitumor effects of ICIs, particularly anti-CTLA-4 antibody and chemotherapy (CDDP or GEM), in murine MPM models [33–35], no report described the combination of anti-PD-1 antibody and standard chemotherapy for MPM.

In the present study, we first demonstrated that the combination of anti-PD-1 antibody with chemotherapy (CDDP + PEM) enhanced the antitumor effects against MPM in mice. This combined effect was associated with the marked increase of CD8 + T cells in tumors, probably mediated by the reduction in intratumoral MDSCs, although the number of Treg cells actually increased. The infiltration of CD8 + T cells, which play a role in eliminating tumors as CTLs, into tumors is known to be critical in inducing effective tumor immunity in mice [36,37] and associated with a better prognosis among patients with MPM [38–40]. Regarding how MDSCs infiltrate tumors, the involvement of VEGF has been particularly well studied [41–43]. It was shown that intratumoral MDSCs express VEGF receptor 1 and 2, and the migration and differentiation of MDSCs were augmented by VEGF signaling [41]. Therefore, reducing the VEGF expression in the tumor is considered to be a useful strategy for inhibiting the migration of MDSCs and in turn enhancing the efficacy of immunotherapy. As shown in the present and previous studies [44], CDDP may be a promising chemotherapeutic agent, as it had the potent effect of reducing the VEGF concentration in the tumor. In addition, PEM was found to significantly decrease VEGF production by MPM cells, although the level of reduction was relatively slight compared to that induced by CDDP. However, further analyses will be required in order to clarify how PEM modulates tumor immunity against MPM. Based on our results together with previous reports [45,46], targeting MDSCs is expected to be a promising combined therapy with anti-PD-1/PD-L1 antibody against various tumors.

Another interesting finding in the present study is the increased number of Treg cells after treatment with anti-PD-1 antibody in both the AB1-HA and LLC models. This is not surprising because recent reports have shown that antigen-specific Treg cells also expanded in tumors on treatment with anti-PD-1 antibody [36,47]. In addition, in contrast to CPA and GEM, it has been reported that CDDP did not reduce Treg cells [48] and that PEM actually increased their population in murine MPM models [49]. A combination to reduce the number of intratumoral Treg cells with CDDP/PEM + anti-PD-1 antibody may be more effective. A further study exploring the role of Treg cells in MPM therapy will be required. Furthermore, the present study is limited in that the role of effector CD4 + T cells was not analyzed, although the total CD4 + T cell count was further augmented by combined therapy with anti-PD-1 therapy and chemotherapy. Although the reason is not clear, the recent reports suggested the contribution of PEM treatment and/or the reduction of MDSCs. Schaer et al. showed that treatment with PEM increased the number of both CD4+ and CD8 + T cells in tumors [50]. Shi et al. reported that depletion of MDSCs with anti-Gr-1 antibody increased the number of both T cells [51]. These data suggest that combination with PEM or the decreased number of MDSCs through the reduction of VEGF production may contribute the increased number of CD4+ and/or CD8 + T cells in the present study.

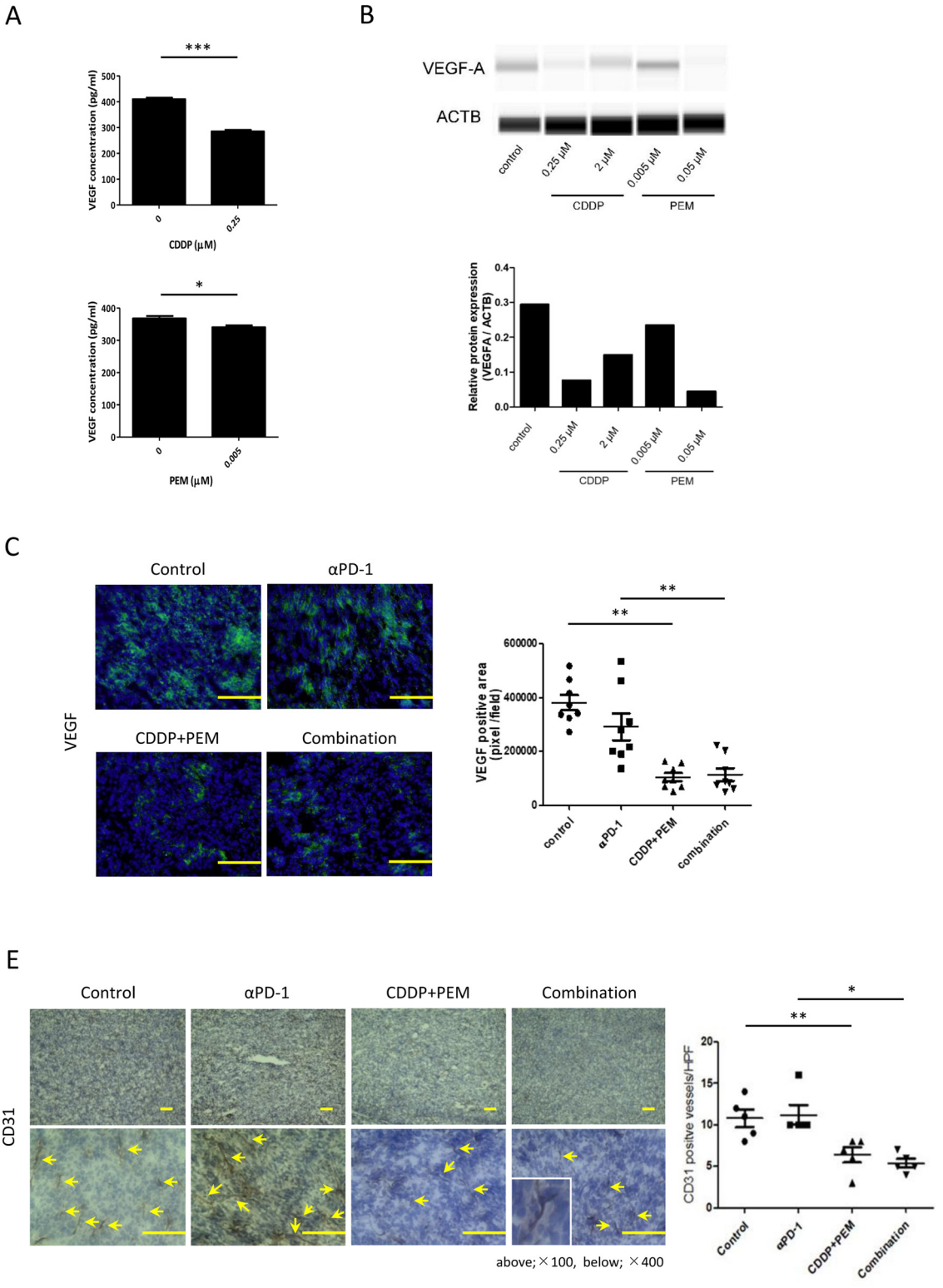

Fig. 5. The assessment of the VEGF expression in AB1-HA cells *in vitro* and *in vivo*. (A) The VEGF concentration in the culture supernatant of AB1-HA cells treated with 0.25 μM of CDDP or 0.005 μM of PEM for 48 h was determined by an ELISA. (B) Western blot analysis of VEGF in tumors treated with combined therapy of CDDP/PEM and anti-PD-1 antibody. Tumor homogenates in each treatment groups were examined the expression of VEGF with the Simple Western™ System. The data also expressed the relative expression of VEGF-A/ACTB ratio. (C) Immunofluorescence analysis of VEGF expression in each treatment group of the AB1-HA subcutaneous tumor. Scale bar, 500 μm. Magnification, ×400, x1600 (inside window). Data are shown as the mean ± SEM. $*P < 0.01$, $**P < 0.05$. (D) Immunostaining of tumor vessels with anti-CD31 antibody. Subcutaneous AB1-HA tumors treated with anti-PD-1 antibody, CDDP + PEM or their combination were harvested on day 28 and stained with anti-CD31 antibody. The arrows in the pictures indicate the positive cells for CD31 staining. Scale bar, 500 μm. Magnification, ×100, ×400, x1600 (inside window). Data are shown as mean ± SEM. $*P < 0.01$, $**P < 0.05$.

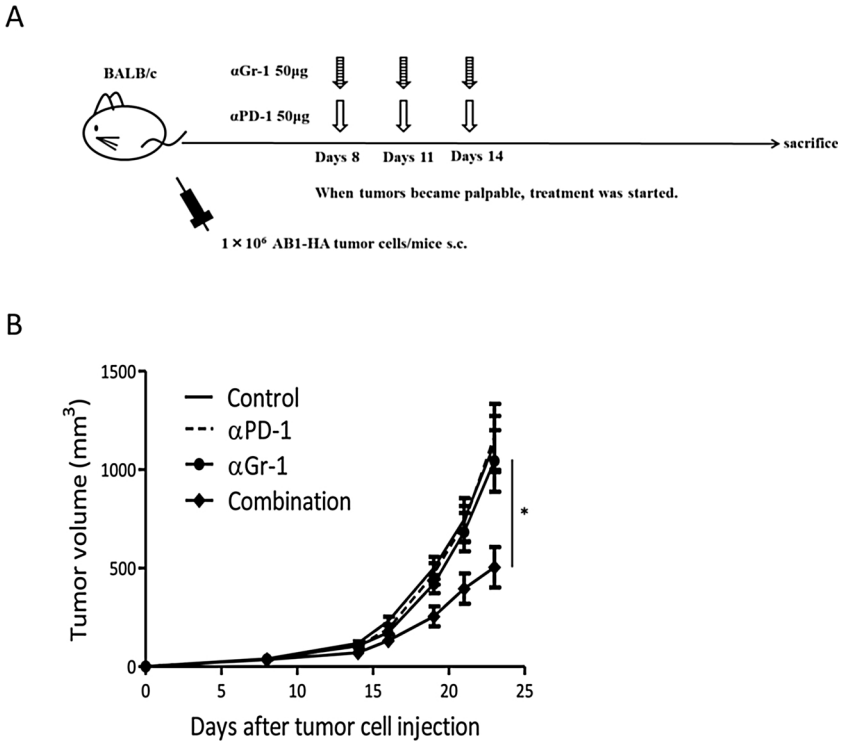

Fig. 6. Anti-tumor effects of anti-PD-1 antibody combined with anti-Gr-1 antibody against AB1-HA tumor. (A) The treatment schedule for the combination therapy. (B) Anti-tumor effects of suboptimal dose of anti-PD-1 antibody combined with anti-Gr-1 antibody. When AB1-HA tumors became palpable, mice were treated with 50 μg of anti-PD-1 antibody alone, 50 μg of anti-Gr-1 antibody alone and their combination on days 8, 11 and 14. $^{*}P < 0.05$. Data are shown as the mean ± SEM.

At present, combination immunotherapy of pembrolizumab, durvalumab or atezolizumab with CDDP + PEM is being evaluated for its efficacy against MPM in phase I and II clinical trials [1,3]. In our preclinical study, we demonstrated the efficacy of combined immunotherapy with anti-PD-1 antibody and chemotherapy (CDDP + PEM) in an MPM model. Our results may provide immunological rationale to those clinical trials and strongly support the early completion of those studies. In the future, we should explore the best anti-cancer drugs or regimens for enhancing the anti-tumor effects of anti-PD-1/PD-L1 antibody in combination against MPM.

Transparency document

The Transparency document associated with this article can be found in the online version.

CRediT authorship contribution statement

Kenji Otsuka: Investigation, Formal analysis, Writing - original draft. **Atsushi Mitsuhashi:** Data curation. **Hisatsugu Goto:** Conceptualization, Methodology. **Masaki Hanibuchi:** Conceptualization, Methodology. **Kazuya Koyama:** Data curation. **Hirohisa Ogawa:** Data curation. **Hirokazu Ogino:** Data curation. **Atsuro Saijo:** Data curation. **Hiroyuki Kozai:** Data curation. **Hiroto Yoneda:** Data curation. **Makoto Tobiume:** Data curation. **Masatoshi Kishuku:** Data curation. **Keisuke Ishizawa:** Data curation. **Yasuhiko Nishioka:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

Yasuhiko Nishioka reports research fees paid to his institution and personal fees from Nippon Boehringer Ingelheim Co., Ltd., Ono Pharmaceutical Co., Ltd., and Chugai Pharmaceutical Co., Ltd.; personal fees from AstraZeneca K. K.; and research fees paid to his institution from Taiho Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Eisai Co., Ltd., and Pfizer Japan Inc.

For other authors, `Declarations of interest: none'.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2020.05.023.

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