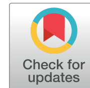

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A Novel Anti-PD-L1 Antibody Exhibits Antitumor Effects on Multiple Myeloma in Murine Models via Antibody-Dependent Cellular Cytotoxicity

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Abstract

Multiple myeloma is a malignant cancer of plasma cells. Despite recent progress with immunomodulatory drugs and proteasome inhibitors, it remains an incurable disease that requires other strategies to overcome its recurrence and non-response. Based on the high expression levels of programmed death-ligand 1 (PD-L1) in human multiple myeloma isolated from bone marrow and the murine myeloma cell lines, NS-1 and MOPC-315, we propose PD-L1 molecule as a target of anti-multiple myeloma therapy. We developed a novel anti-PD-L1 antibody containing a murine immunoglobulin G subclass 2a (IgG2a) fragment crystallizable (Fc) domain that can induce antibody-dependent cellular cytotoxicity. The newly developed anti-PD-L1 antibody showed significant antitumor effects against multiple myeloma in mice subcutaneously, intraperitoneally, or intravenously inoculated with NS-1 and MOPC-315 cells. The anti-PD-L1 effects on multiple myeloma may be related to a decrease in the immunosuppressive myeloid-derived suppressor cells (MDSCs), but there were no changes in the splenic MDSCs after combined treatment with lenalidomide and the anti-PD-L1 antibody. Interestingly, the newly developed anti-PD-L1 antibody can induce antibody-dependent cellular cytotoxicity in the myeloma cells, which differs from the existing anti-PD-L1 antibodies. Collectively, we have developed a new anti-PD-L1 antibody that binds to mouse and human PD-L1 and demonstrated the antitumor effects of the antibody in several syngeneic murine myeloma models. Thus, PD-L1 is a promising target to treat multiple myeloma, and the novel anti-PD-L1 antibody may be an effective anti-myeloma drug via antibody-dependent cellular cytotoxicity effects.

Key Words: PD-L1, Multiple myeloma, Antibody-dependent cellular cytotoxicity (ADCC), Myeloid-derived suppressor cell (MDSC), Lenalidomide

INTRODUCTION

Multiple myeloma is a type of cancer characterized by the uncontrolled clonal expansion of malignant plasma cells within the bone marrow (Palumbo and Anderson, 2011). Standard therapeutic regimens with FDA-approved substances, such as immunomodulatory drugs (lenalidomide and pomalidomide) and proteasome inhibitors (bortezomib) (Richardson *et al*., 2018), have been established, but multiple myeloma remains an incurable disease. Thus, further study of novel drug development is required.

Programmed death-ligand 1 (PD-L1), also known as B7 homolog 1 (B7-H1) or CD274, is an immune checkpoint molecule that transmits an inhibitory signal to counterpart cells via the binding of PD-1 and CD80 (Zou and Chen, 2008). PD-L1 is expressed in various cells, including antigen-presenting cells (dendritic cells and macrophages), activated B cells, and other nonlymphoid tissue cells, including the heart, lung, liver, and kidney (Iwai *et al*., 2002). PD-L1 has also been detected in several types of tumor cells, including hepatocellular carcinoma, breast cancer, pancreatic cancer, colon cancer, and blood cancer. Interestingly, most multiple myeloid cells express high

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levels of PD-L1 on their surface (Liu *et al*., 2007), and the blockade of the PD-1/PD-L1 interaction inhibited tumor growth in a syngeneic murine model of myeloma (Iwai *et al*., 2002).

The blockade of PD-L1 also mitigated immunosuppression mediated by the myeloid-derived suppressor cells (MDSCs), which are a heterogeneous population of immature myeloid cells that have different PD-1/PD-L1 expression levels depending on the tumor type (Deng *et al*., 2014; Nam *et al*., 2019; Lim *et al*., 2020). MDSCs suppress innate and adaptive immune responses in the tumor microenvironment (Youn *et al*., 2008; Ko and Kim, 2016) but the interactive relationships between the immune cells, PD-L1-expressing cancer cells, and MDSCs in the tumor environment have never been investigated. Although compounds such as all-trans retinoic acid induced the conversion of MDSCs into immunogenic dendritic cells (Lee *et al*., 2012), patterns of PD-L1 expression during this process were not reported. Thus, the role of PD-L1 in the immunosuppressive function of MDSCs requires further investigation.

In this study, we developed a new anti-PD-L1 antibody (Ab) that binds to mouse and human PD-L1. The Ab significantly inhibited syngeneic myeloma cell growth in mice. There were no significant changes in the MDSC composition of tumor-bearing mice following anti-PD-L1 treatment, but the anti-PD-L1 antibody induced antibody-dependent cellular cytotoxicity (ADCC)-associated myeloma cell death. We sought to validate the synergistic efficacy of anti-PD-L1 Ab treatment with lenalidomide in the murine model of multiple myeloma and observed only PD-L1-driven inhibition of tumor growth (lenalidomide itself was not effective in the mouse model). Collectively, our results show that the newly developed anti-PD-L1 Ab may be a therapeutic candidate for multiple myeloma, though further studies are required to differentiate it from other anti-PD-L1 therapeutic Abs.

MATERIALS AND METHODS

Cell lines and reagents

NS-1 and MOPC-315 murine myeloma cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The NS-1 cells were maintained in RPMI 1640 medium, and the MOPC-315 cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% of fetal bovine serum and 1% anti-anti solution. Both cell lines were sub-cultured every 2-3 days. Lenalidomide (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO, and the stock solution was stored at –70°C. Phycoerythrin-(PE) conjugated anti-mouse PD-L1 antibody was purchased from BD Bioscience (San Jose, CA, USA) and used as a control antibody for the mouse PD-L1 expression assays.

Construction of the novel, mouse IgG2a-based, anti-PD-L1 antibody

A novel anti-PD-L1 antibody with human antibody residues (Choi *et al*., 2020) was engineered to have mouse immunoglobulin G subclass 2a (IgG2a) and kappa domains to avoid mouse anti-human antibody immune responses while maintaining the mice effector functions. The chimeric antibody heavy and light chains were constructed as fusions containing the mouse IgG2a and kappa constant domains, but their original variable regions, V_H and V_L, were maintained for their antigen binding characteristics. Overlap polymerase chain reaction (PCR) was used to assemble the human variable domains and mouse constant domains, and the chimeric heavy and light chains were amplified and cloned into the pCEP4 mammalian expression vector. The chimeric anti-PD-L1 antibody was produced in the ExpiCHO expression system (Thermo, Carlsbad, CA, USA), and the transfection and cell culture were performed with the 'MAX titer' option, according to the manufacturer's recommendation. After 12 days, the culture medium was collected, and the antibody was purified by affinity column chromatography on protein A agarose (GenScript, Piscataway, NJ, USA).

Preparation of human peripheral blood mononuclear cells (PBMC) and bone marrow cells

The study specimens were obtained from multiple myeloma patients who underwent bone marrow aspiration at the Korea University Anam Hospital (Seoul, Korea) . The study protocol was approved by the Institutional Review Board of the Korea University Medical Center (Seoul, Korea), and the patients provided written informed consent. All methods were performed according to the relevant guidelines and regulations (IRB No. 2018AN0150). Aspirated bone marrow and peripheral blood samples were diluted to a ratio of 1:1 with phosphate-buffered saline (PBS) and layered over the same original blood volume of Ficoll (Histopaque-1077; Sigma-Aldrich) in a 50 mL conical tube. The specimens were centrifuged at 2,000 rpm for 30 min at room temperature, and the upper layer of the opaque interface (containing the mononuclear cells) was aspirated and transferred to a new conical tube. The collected cells were washed with 5 mL of PBS and centrifuged at 1,200 rpm for 10 min at room temperature.

Animal experiments

Six-week-old female Balb/c mice were purchased from KOATECH (Pyeongtaek, Korea). The mice were maintained under specific pathogen-free conditions for 1 week in the experimental facilities at Kangwon National University (Chuncheon, Korea), where they received sterilized food and water ad libitum and were housed at 20-22°C on a 12 h light/dark cycle. All of the animal experiments were performed according to the approved guidelines of the Institutional Animal Care and Use Committee of Kangwon National University (KW-140811-2). To establish a mouse myeloma model, 7- or 8-week-old mice were challenged with 5×10^6 NS-1 cells (subcutaneously or intraperitoneally) or 10^7 of NS-1 or MOPC-315 cells (intravenously). Tumor length, height, and width were measured with calipers, and the tumor volume was calculated as $1/6\pi\times$length (mm)×height (mm)×width (mm). To evaluate the anti-myeloma efficacy of lenalidomide and the anti-human/murine PD-L1 antibody, 10 mg/kg of Lenalidomide (every day) and 5 mg/kg of PD-L1 antibody (every 2-3 days) was intraperitoneally injected. Mouse body condition was scored as 0 (normal), 1 (rough or harsh hair), 2 (partial paralysis of the hind leg or emergence of a cancer nodule near the skin), 3 (complete paralysis of the hind leg or a larger tumor mass), 4 (complete paralysis of the hind leg and reduction of motion), or 5 (death).

Flow cytometry

To analyze PD-L1 expression on the cell surface, 5×10^5 of NS-1 and MOPC-315 cells were stained with PE-conjugated anti-mouse PD-L1 (BD Bioscience) and Alexa Flour 488-con-

jugated anti-human/mouse-PD-L1 antibodies for 15 min 4°C in flow cytometry staining buffer (FACS; PBS supplemented with 1% fetal bovine serum (FBS), 2 mM EDTA). After staining, the cells were washed with 1 ml of FACS buffer and analyzed on a FACSVerse instrument (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For analysis of the human PBMC and bone marrow cells, frozen cell stock vials were thawed at 37°C, and approximately 10^5 PBMC or bone marrow cells were stained with PE-Cy7-conjugated anti-CD3 antibody, brilliant violet 421 (BV421)-conjugated anti-CD138 antibody, APC-conjugated anti-human PD-L1 antibody (29E.2A3), or unconjugated anti-human/murine PD-L1 antibody. To detect the anti-human/murine PD-L1 antibody, a second staining was conducted with Alexa Flour 647-conjugated anti-mouse IgG antibody. After staining, the cells were washed with 1 mL of FACS buffer and analyzed on the FACSVerse instrument. Flow cytometry experiment to detect MDSCs were conducted as reported previously and some parts of procedures were revised (Song *et al.*, 2018). Briefly, spleen isolated from tumor-bearing mice were crushed and RBCs were lysed. Splenoycytes were stained with BV421-conjugated anti-CD11b antibody, PE-Cy7-conjugated anti-Ly6G antibody, APC-conjugated anti-Ly6C antibody. All of the flow cytometry results and graphs were generated in FlowJo (FlowJo Inc., Ashland, OR, USA).

Immunofluorescence staining

The formalin-fixed paraffin-embedded specimens were sectioned (4-5 μm) and placed on slides, which were deparaffinized and then rehydrated. Antigen retrieval was performed by boiling in a pressure cooker for 10 min with a sodium citrate buffer (pH 6.0), and permeabilization was performed with 0.5% Triton X-100. The specimens were blocked using 5% normal donkey serum for 1 h at room temperature and then incubated overnight at 4°C with the primary antibodies targeting CD138 (1:100; R&D System, Minneapolis, MN, USA) and PD-L1 (ABM4E54, 1:100; Abcam, Cambridge, UK). The samples were then incubated with the fluorochrome-conjugated secondary antibodies for the CD138 (Alexa Fluor 488, 1:200; Invitrogen, Carlsbad, CA, USA) and PD-L1 tests (Alexa Fluor 647, 1:200; Invitrogen) at room temperature for 1 h. Isotype-matched antibodies were used as the negative controls, and the nuclei were highlighted using DAPI (4′,6-diamidino-2-phenylindole) mounting medium (ProLong™ Diamond Antifade Mountant with DAPI; Invitrogen). The samples were imaged on an automated fluorescence microscope (200× magnification; EVOS FL Auto; Life Technologies) and processed in Celleste™ image analysis software (Invitrogen).

Antibody-dependent cytotoxicity assay (ADCC assay)

MOPC-315 cells and splenocytes were seeded in a 96-well plate (10^4 cells/well); the cells were treated with 1, 5, 50, 100, and 200 μg/ml drugs (lenalidomide and anti-PD-L1 antibody) and incubated for 4 h in an incubator at 37°C and 5% carbon dioxide (CO_2). After incubation, 10 μL of cell counting kit-8 (Cell Counting Kit-8, Dojindo Co., Kumamoto, Japan) were added to each well and incubated for an additional 2 h. The absorbance at 450 nm was measured by a SpectraMax i3 microplate reader (Molecular Devices, San Jose, CA, USA).

To isolate mouse peripheral blood mononuclear cells, mouse blood was collected by heart puncture in tubes containing EDTA. Spleen, mesenteric, inguinal, axillary, and cervical lymph node samples were obtained from naïve C57Bl/6 mice and mechanically strained through a 100 μm nylon strainer. The blood and strained cells were mixed, layered on 3 ml of Histopaque®-1077 (Sigma-Aldrich), and gently centrifuged for 30 min at room temperature with minimum acceleration and deceleration. Interphase cells were collected and washed 3 times with cold PBS, and the red blood cells (RBCs) were lysed with RBC lysis buffer. PBMCs (2-3×10^6) were cultured in complete RPMI medium supplemented with 20 ng/mL of murine IL-2 for 48 h.

The MOPC-315 cells were equally divided and stained with 0.5 μM and 5 μM of cell trace violet (CTV, Invitrogen) for 10 mins at 37°C. The MOPC-315 cells stained with 5 uM of CTV were reacted with 0.1 μg/mL of anti-PD-L1 Ab for 30 min at 4°C; the 0.5 uM CTV-stained cells were an internal control (no antibody added). The cells were washed 3 times with cold PBS and mixed equally, and the mixed MOPC-315 cells (5×10^4) were co-cultured with IL-2 primed mouse PBMC for 4 h at 37°C. After incubation, the cells were analyzed on the FACSVerse instrument. The specific lysis ratio was calculated as r (ratio)=(% CTV^{high}/CTV^{low}), and the percent lysis (%) was calculated as lysis %=[1–(*r*_*unpulsed*_/*r*_*pulsed*_)]×100.

Statistical analysis

Statistical analyses were performed in Graphpad Prism 5 (GraphPad Software, LLC, San Diego, CA, USA). Unpaired two-tailed Student's *t*-tests were used when the data had a Gaussian distribution with similar variances, and Mann-Whitney U tests were used when the data deviated from a Gaussian distribution. One-way analysis of variance (ANOVA) was

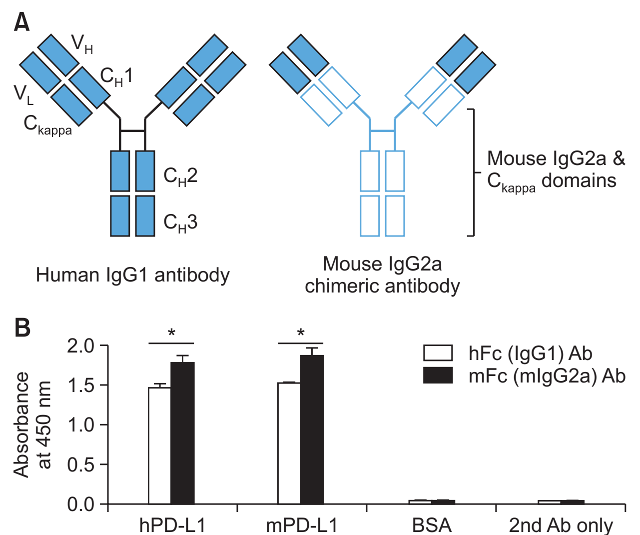

Fig. 1. Construction of the chimeric anti-PD-L1 antibody against human and mouse PD-L1 antigens. (A) Schematic presentation of the chimeric, mouse IgG2a-based antibody structure compared to the original novel anti-PD-L1 antibody with full human IgG1 sequences. (B) ELISA was performed with a purified original human antibody (hIgG1) and a chimeric antibody (mIgG2a) on 96-well microtiter plates coated with human and mouse PD-L1 antigens. To comply with the detection of human and mouse antibody constant domains of each antibody, horseradish peroxidase- (HRP) conjugated goat anti-human kappa light chain IgG (1:5,000) and HRP-conjugated goat anti-mouse IgG (1:400) were used as secondary antibodies. Bovine serum albumin (BSA) was used as a background control, *$p<0.05$.

used for multigroup comparisons, and survival (Kaplan-Meier) curves were compared with the log-rank test. The threshold for statistical significance was $p<0.05$, with 95% confidence intervals for all of the analyses.

RESULTS

Construction of the mouse IgG2a-based, anti-PD-L1 antibody

To validate a novel therapeutic approach to treat multiple myeloma, we constructed a chimeric, mouse-compatible version of a newly generated, fully human anti-PD-L1 Ab that maintained the human V_H and V_L domains and fused with the mouse IgG2a and kappa constant domains to avoid mouse anti-human IgG1 immune responses. The Ab retained the effector functions, such as the antibody-dependent cellular cytotoxicity (ADCC) and the complement-dependent cytotoxicity (CDC) in mice (Fig. 1A). The original anti-PD-L1 Ab was isolated through phage display screening using a naïve human antibody library and had cross-species reactivity to both murine and human PD-L1. It also showed strong *in vitro* neutralizing activity on the PD-1/PD-L1 interaction and highly efficacious antitumor growth inhibition activities in a syngeneic *in vivo* mouse model (Choi *et al*., 2020). The new chimeric mouse IgG2a-based antibody was expressed from the ExpiCHO system and purified using protein A column chromatography. The binding property of the chimeric antibody was tested via an enzyme-linked immunosorbent assay (ELISA) - it maintained its binding capabilities against mouse and human PD-L1 antigens (Fig. 1B).

Expression of PD-L1 in human and murine multiple myeloma

PD-L1 was expressed on the plasma membrane of $CD138^+$ cells from multiple myeloma patients, and analysis of the bone marrow cells of multiple myeloma patients revealed $CD138^+$ cells with high PD-L1 expression (Fig. 2A). The expression of PD-L1 on $CD138^+$ cells from multiple myeloma patients was detected with flow cytometry, and the mean fluorescence index of PD-L1 was compared to isotype control Ab staining (Fig. 2B). The expression of PD-L1 on $CD138^+$ cells has been associated with bad patient prognosis (Yousef *et al*., 2015), and recent studies have suggested that multiple myeloma cells (but not normal plasma cells) from multiple myeloma patients have elevated PD-L1 levels (Liu *et al*., 2007; Dhodapkar *et al*., 2015). Several multiple myeloma cells, including SP2/0, P3U1, X63, J558L, and PAI, express PD-L1 on their surface (Iwai *et al*., 2002), so we assessed if NS-1 and MOPC-1 also express PD-L1. Staining with commercially available antibody labeled fluorescence dyes showed that PD-L1 was highly expressed on the surface of NS-1 and MOPC-315 cells (Fig. 2C). Stain-

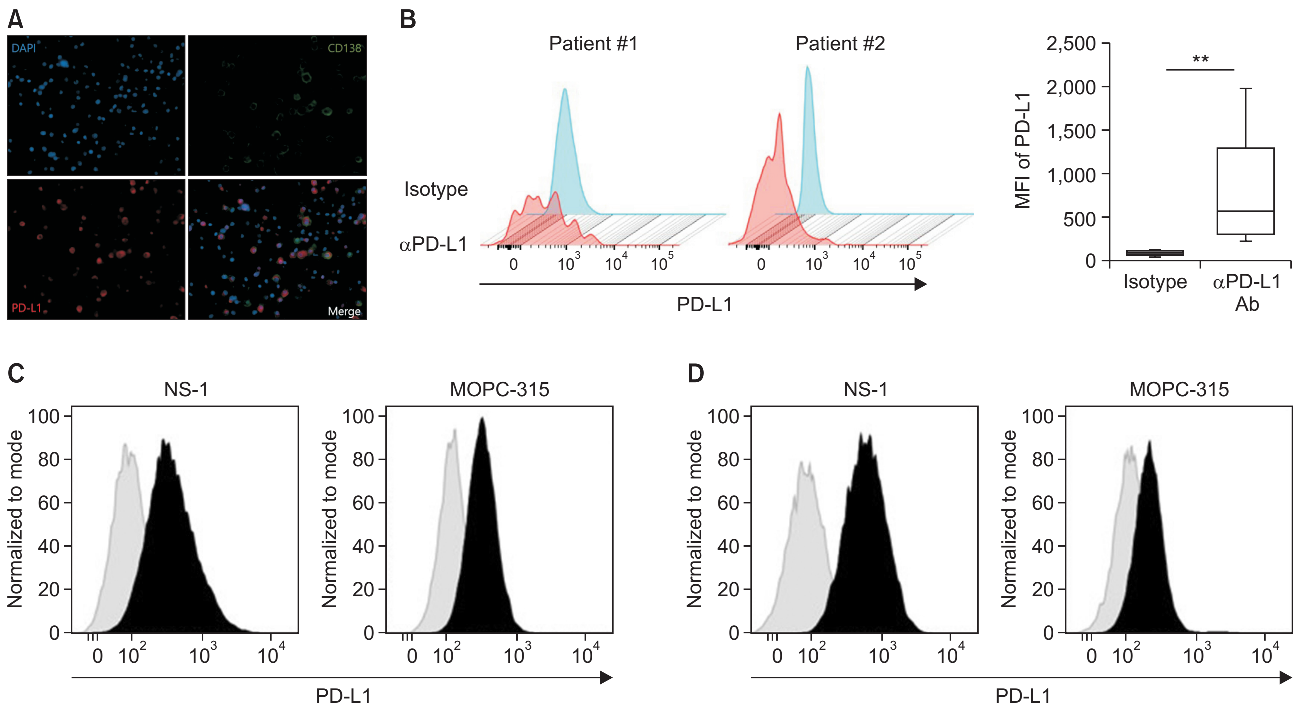

Fig. 2. PD-L1 expression in multiple myeloma and murine myeloma cell lines. (A) Cell block micro-sections made with the bone marrow of multiple myeloma patients were stained with anti-PD-L1 and anti-CD138 antibodies. Representative fluorescence-microscope pictures of DAPI (blue), CD138 (green), PD-L1 (red), and merged channels are shown. (B) PD-L1 expression in $CD138^+$ cells was analyzed by flow cytometry in bone marrow cell isolates from multiple myeloma patients (n=5). The bone marrow cells were stained with a commercial anti-human PD-L1 antibody (clone name, 29E.2A3). (C) PD-L1 expression in mouse myeloma cell lines (NS-1 and MOPC-315) was detected with a commercial anti-mouse PD-L1 antibody (clone name, MIH5). A representative flow cytometric histogram of PD-L1 expression (black) and isotype control (grey) is shown. (D) PD-L1 expression in mouse myeloma cell lines (NS-1 and MOPC-315) was detected with an Alexa Fluor 488-conjugated, newly developed chimeric PD-L1 antibody. A representative flow cytometric histogram of PD-L1 expression (black) and isotype control (grey) is shown. Significance compared to the control, $^{**}p<0.01$.

ing with commercially available anti-PD-L1 antibody labeled fluorescence dyes or a newly developed monoclonal antibody with Alex Fluor 488-labeled secondary Ab successfully demonstrated that PD-L1 was highly expressed on the surface of NS-1 and MOPC-1 cells (Fig. 2D). We also demonstrated that the anti-PD-L1 Ab bound to PD-L1 on NS-1, MOPC-1, CD138$^+$ cells from multiple myeloma patients. These results are consistent with previous data showing PD-L1 expression on multiple myeloma cells in humans and mice and suggest possible immunotherapy with the newly developed anti-PD-L1 Ab for the treatment of multiple myeloma.

Blockade of PD-L1 reduces tumor development in murine multiple myeloma models

To assess the therapeutic effects of the newly developed anti-PD-L1 Ab *in vivo*, we adopted several murine models. We subcutaneously inoculated NS-1 cells (5×10^6 cells per mouse) into the left flank of BALB/c mice; the NS-1 tumor grew rapidly and all mice reached about 100 mm^3 of tumor burden within 14 days. We intraperitoneally treated the mice with 100 μg of anti-PD-L1 Ab or an isotype control Ab every two days for five total treatments, starting at 14 days after tumor inoculation. We observed a significant delay in tumor growth in the mice that received anti-PD-L1 Ab, and 60% of the mice inoculated with NS-1 became tumor-free at 24 days post tumor challenge (Fig. 3A, 3B). Intraperitoneal injection of the NS-1 cells resulted in a solid tumor in the abdomen with increased body weight after 15 days of tumor challenge. However, the effect was reversed by treatment with anti-PD-L1 Ab (Fig. 3C, 3D).

Multiple myeloma causes cancer cell accumulation in the bone marrow, so we adopted intravenous injection models of multiple myeloma cells. Intravenous injection of the NS-1 cells into BALB/c mice (1×10^7 cells per mouse) induced lethality within 80 days after tumor challenge, whereas anti-PD-L1 Ab treatment (5 times, 2-day intervals) extended the survival (Fig. 4A). Likewise, intraperitoneal injection of anti-PD-L1 Ab increased mouse survival compared to the MOPC-315 cells-injected mice treated with isotype control Ab (Fig. 4C). We also

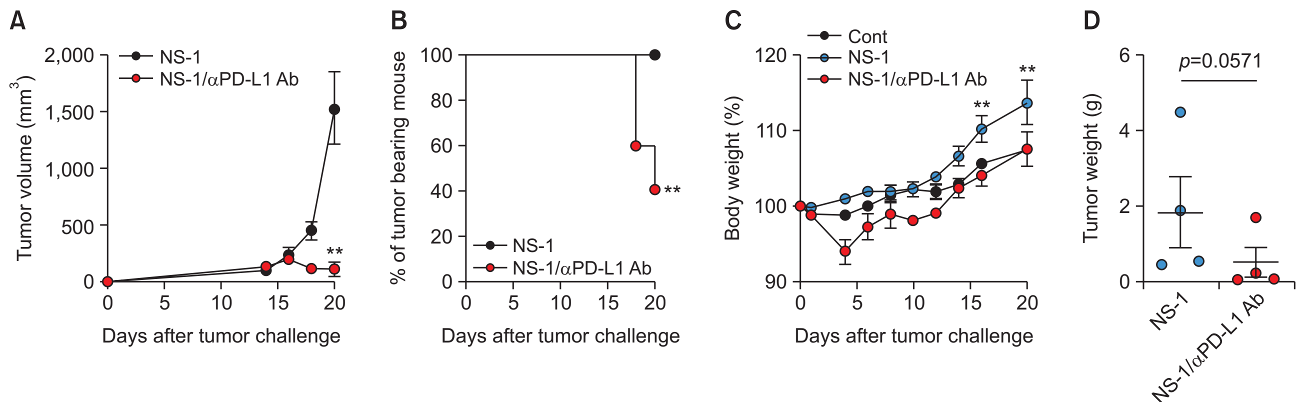

Fig. 3. Anti-myeloma efficacy of the anti-PD-L1 Ab treatment in the mouse myeloma model. (A, B) Six-week-old female BALB/c mice were subcutaneously injected with 5×10^6 cells of NS-1 mouse myeloma cells. novel chimeric PD-L1 antibody (100 μg) was intraperitoneally injected into each mouse 15 days after tumor challenge, every other day. Tumor volumes were measured every other day to monitor tumor development. (C) Six-week-old female BALB/c mice were intraperitoneally injected with 5×10^6 cells of NS-1 mouse myeloma cells. The next day, 100 μg of newly developed chimeric PD-L1 antibody was intraperitoneally injected into each mouse, every other day. (D) The weight of the tumor isolated from the peritoneal cavity of the NS-1 bearing mice. Significance compared to the control, $^{**}p$<0.01.

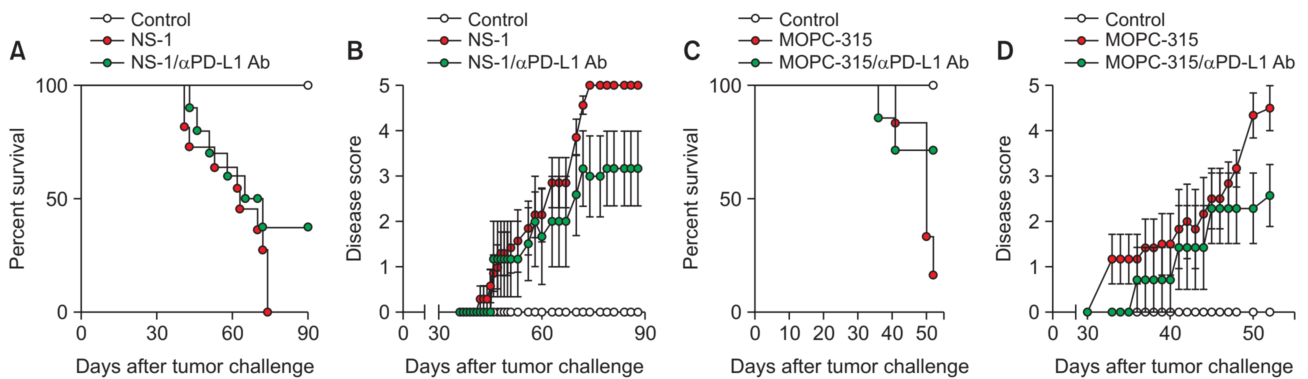

Fig. 4. Anti-PD-L1 therapy attenuated the progression of myeloma in the mouse intravenous model. NS-1 and MOPC-315 myeloma cells (10^7) from the BALB/c mice were injected into the tail vein. Thirty days after the tumor challenge, 100 μg of novel chimeric PD-L1 antibody was administered by intraperitoneal injection. The survival and phenotypical changes were scored as described in the materials and methods section. (A) Percent survival and (B) Disease score of NS-1-bearing mice treated with anti-PD-L1 Ab. (C) Percent survival and (D) Disease score of MOPC-315-bearing mice treated with anti-PD-L1 Ab.

visually scored the body condition of the mice, based on the general appearance, occurrence of paralysis or solid tumors, and morbidity (as described in the materials and methods). Interestingly, treatment with anti-PD-L1 Ab significantly ameliorated the condition of the NS-1- and MOPC-1-inoculated mice (Fig. 4B, 4D). Together, these results suggest that the newly generated anti-PD-L1 Ab successfully inhibited the growth of multiple myeloma in syngeneic murine models.

Combined treatment with anti-PD-L1 Ab and lenalidomide was not more effective and did not significantly inhibit the generation of MDSCs

Lenalidomide is an immunomodulatory drug used to treat multiple myeloma. We investigated if the combination of lenalidomide and anti-PD-L1 Ab has synergistic effects in a murine myeloma model. Groups of mice were subcutaneously injected with MOPC-315 cells (5×10^6 cells per mouse) and treated with lenalidomide (10 mg/kg) and anti-PD-L1 Ab (100 μg/mouse). Starting from day 14, lenalidomide was administered every day, and anti-PD-L1 Ab was injected every other day. The anti-PD-L1 Ab treatment significantly inhibited tumor growth, but we did not observe any decrease in tumor size in the mice treated with lenalidomide (compared to the PBS-treated control group; Fig. 5A). Furthermore, there were no additive antitumor effects when lenalidomide was combined with anti-PD-L1 Ab (Fig. 5A). These results highlight the an-

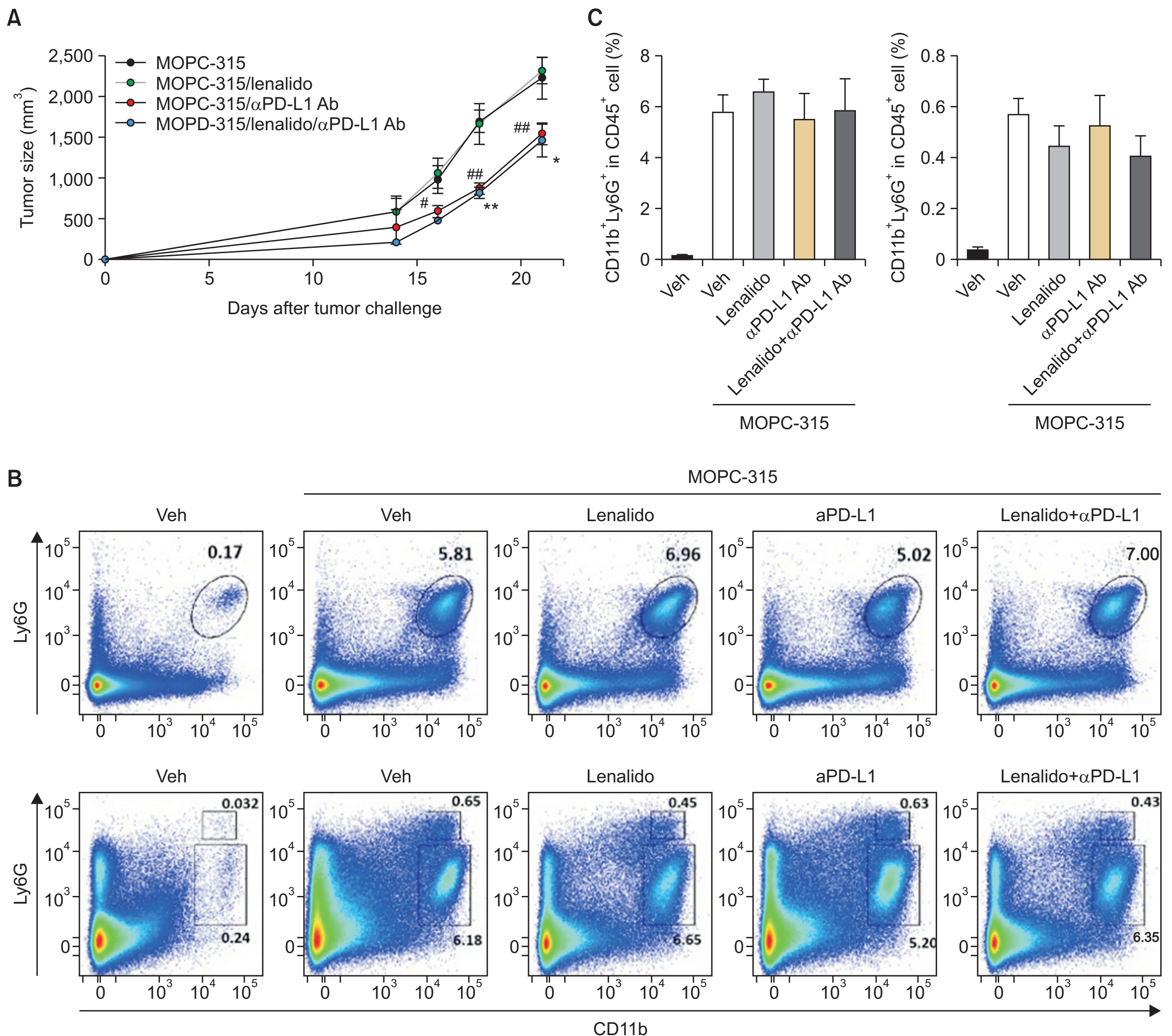

Fig. 5. Lenalidomide and anti-PD-L1 Ab combination therapy was not effective in the subcutaneous mouse myeloma model. MOPC-315 cells (1×10^6) were injected into the subcutaneous cavity of BALB/c mice. Fourteen days after the tumor challenge, 10 mg/kg of lenalidomide and the newly developed chimeric PD-L1 antibody were administered intraperitoneally. (A) The combined treatment of lenalidomide and anti-PD-L1 antibody to the MOPC-315 tumor-bearing mice. The tumor growth curve is depicted. (B, C) Representative flow cytometry plots. Significance compared to the MOPC-315 and MOPC-315/αPD-L1 groups, *p<0.05, **p<0.01. Significance compared to the MOPC-315/Lenalido and MOPC-315/Lenalido/αPD-L1 groups, #p<0.05, ##p<0.01.

titumor effects of anti-PD-L1 Ab on the multiple myeloma in murine models but do not reveal any synergistic effects of anti-PD-L1 and lenalidomide.

Myeloid-derived suppressor cells (MDSCs) are a mixed population of immature myeloid cells, containing neutrophils, monocytes, and immature dendritic cells (Lee *et al*., 2016). In tumor-bearing mice, there are several subsets, such as neutrophilic MDSCs with a $CD11b^{+}Ly6G^{+}$ phenotype and monocytic MDSCs with a $CD11b^{+}Ly6C^{+}$ phenotype (Lee *et al*., 2016). We assessed if multiple myeloma in mice induces the generation of MDSCs. The subcutaneous injection of MOPC-315 significantly increased splenic MDSCs (neutrophilic and monocytic MDSCs), but anti-PD-L1 Ab treatment did not reduce the MDSC levels in the spleens of the tumor-bearing mice (Fig. 5B, 5C). Also, there was only marginal PD-L1 expression on the MDSCs from the MOPC-315 tumor-bearing mice (Fig. 5B, 5C). Thus, the antitumor effects of anti-PD-L1 Ab in the multiple myeloma model may not be due to the direct effects of PD-L1 expression on MDSCs.

Anti-PD-L1 Ab induces antibody-dependent cellular cytotoxicity-mediated growth inhibition of multiple myeloma

As another antitumor mechanism of anti-PD-L1 Ab, we assessed if the Ab can mediate ADCC on multiple myeloma cells. ADCC plays an important role in the antitumor activities of several monoclonal antibodies targeting cancer (Giles *et al*., 2019), so we assessed the effects of anti-PD-L1 Ab on the *in vitro* ADCC activity of mouse splenocytes against multiple myeloma cells (Fig. 6A). The newly developed anti-PD-L1 antibody was treated with a co-culture of MOPC-315 cells and splenocytes; 4 h after the antibody treatment, MOPC-315 cell viability was significantly reduced at higher concentrations of the anti-PD-L1 Ab treatment (>100 μg/mL; Fig. 6A). In the co-culture system of the anti-PD-L1-bound NS-1 cells and the IL-2-primed mouse PBMCs, the CTV^{high} peak (containing the anti-PD-L1 antibody-bound NS-1 cells) was significantly reduced compared to the anti-PD-L1 antibody-non-bound-NS-1 cells after 4 h incubation (Fig. 6B, 6C). These results suggest that the newly developed anti-PD-L1 Ab induces ADCC effects against NS-1 and MOPC-315 mouse myeloma cells and that the Ab can be used as a more effective treatment for multiple myeloma patients than the PD-1/PD-L1 blockades.

DISCUSSION

Multiple myeloma is a hematological malignancy with clonal plasma cell disorder and represents about one percent of all reported cancers. Recently established standardized therapies with immunomodulatory drugs such as lenalidomide and pomalidomide with proteasome inhibitors (bortezomib, carfilzomib, and ixazomib) markedly improved the outcomes for multiple myeloma patients (Jelinek *et al*., 2018). However, most patients have multiple cycles of remission and relapse with drug resistance, and, consequently, the patients become refractory to conventional therapies. Thus, there is an urgent need for novel therapies based on different modes of action. Recent improvements in the immunotherapeutical approaches, including anti-CD38 monoclonal antibodies and immune checkpoint blockades, have shown promise as novel methods to treat multiple myeloma (Jelinek and Hajek, 2016). The blockade of PD-L1 using antibodies that bind to PD-L1 may confer significant advantages - PD-L1 expression on multiple myeloma cells inhibits tumor-specific cytotoxic T and NK cells via the interaction between PD-L1 and PD-1 and improves the survival of cancer cells and induces myeloma drug resistance (Ishibashi *et al*., 2016).

Contrary to expectations, however, recent clinical reports have shown that the blockade of PD-1 is not effective in multiple myeloma patients (Tremblay-LeMay *et al*., 2018). Moreover, the combined treatment of the PD-1 inhibitor pembrolizumab and standard therapies (e.g., pomalidomide and dexamethasone) failed to increase survival in multiple myeloma patients (Mateos *et al*., 2019; Usmani *et al*., 2019). Thus, a different mode of PD-L1 blockade therapy is needed for multiple myeloma patients.

Targeting PD-L1 has several advantages over a PD-1 inhibitor (Tremblay-LeMay *et al*., 2018). PD-1 expressed on T lymphocytes is involved in the maintenance of peripheral tolerance, and effector T cells ceased their function through activation-induced cell death mediated by PD-1. Interestingly, PD-1 is highly expressed on the T lymphocytes of peripheral blood mononuclear cells from cancer patients. Two types of PD-1 ligands, PD-L1 and PD-L2, are expressed on the surface of several immune cells (e.g., dendritic cells and macrophages). PD-L2 expression is restricted to immune cells, while PD-L1 is found on tumor-associated fibroblasts and several cancer cells in the tumor microenvironment. Several previous

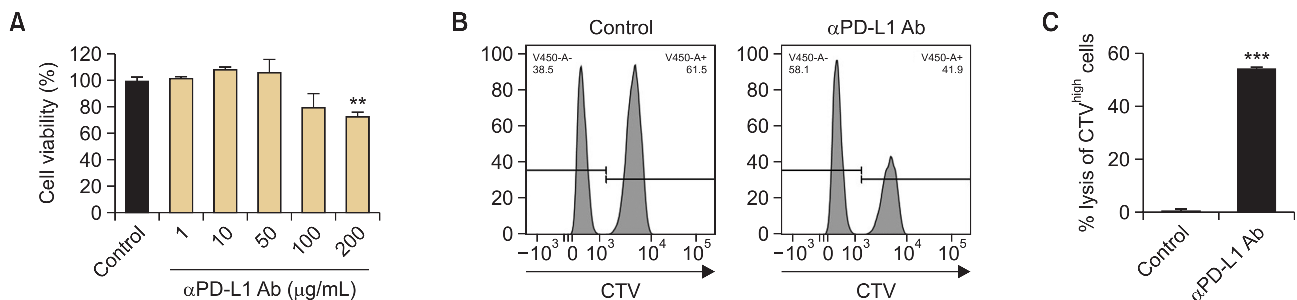

Fig. 6. ADCC effects of the anti-PD-L1 Ab for various myeloma cell lines. (A) MOPC-315 cells and splenocytes from naïve BALB/c mice were co-cultured with different concentrations of anti-PD-L1 Ab. Cell viability was measured via the CCK-8 reagent, and calculated percentages were compared to the PBS-treated control group. (B, C) NS-1 cells were incubated with the anti-PD-L1 Ab to generate anti-PD-L1-bound NS-1 cells. The Ab-bound cells were co-cultured with IL-2-primed mouse PBMC for 4 h. The percent (%) lysis of NS-1 cells is depicted. $^{**}p<0.01$, $^{***}p<0.001$.

studies have reported PD-L1 expression in multiple myeloma cells, but not in the plasma cells of healthy donors (Tamura *et al*., 2013).

The blockade of PD-L1 on tumor cells may be beneficial via intrinsic and extrinsic effects through the inhibition of PD-1/PD-L1 binding. The latter helps T and NK cells respond to cancer cells by overcoming the tumor-associated immune evasion mechanisms (Ray *et al*., 2015). In contrast, the former features are associated with increased proliferation, decreased apoptosis, and increased migration and invasion of the PD-L1-expressing cancer cells compared to PD-L1-knockdown cells (Tremblay-LeMay *et al*., 2018). In multiple myeloma patients, high PD-L1 expression is closely associated with increased bone marrow infiltration by the cancer cells and a higher incidence of relapse/refractory disease. Thus, the blockade of PD-L1 with anti-PD-L1 Ab may have the added benefit of evading antitumor immunity by the T and NK cells, since the inhibition of PD-L1 on multiple myeloma cells can directly inhibit the growth of cancer cells by attenuating the intrinsic aggressive features via PD-L1 signaling. We also showed that the anti-PD-L1 Ab can induce direct cancer cell death via ADCC, suggesting a complicated mode of action for the Ab. We developed the novel anti-PD-L1 antibody with a murine IgG2a domain based on the previous reports that the IgG2a subclass shows potent ADCC effects (Kipps *et al*., 1985). Although the MDSCs also expressed PD-L1 molecules on their cytoplasmic membrane, our data showed little changes in the splenic MDSC population after anti-PD-L1 antibody treatment. This may be due to lower densities of the surface PD-L1 molecule on the MDSCs - antigen density can influence the degree of ADCC reaction (Velders *et al*., 1998). Our data showed that the murine myeloma cells exhibited high PD-L1 expression, but PD-L1 expression on the MDSCs was relatively low compared to the myeloma cells (data not shown). Thus, the difference in PD-L1 antigen density could not explain the significant changes in the MDSC population. However, there was no association between the induction of PD-L1-dependent ADCC and the restoration of T cell function via PD-L1 blockade, so immunotherapy with this novel anti-PD-L1 antibody is still more promising than other anti-PD-L1 antibodies lacking ADCC functioning. Other therapeutic antibodies on the market including atezolizumab and durvalumab are engineered to eliminate ADCC or CDC effect. This engineering minimize eliminating PD-L1-expressing T cells. Although avelumab is designed to induce ADCC, it is only approved for some cancer including metastatic Merkel cell carcinoma and prolonged overall response rate (Kim, 2017; Giles *et al*., 2019). Safety concerns and activity issues have been raised about the adoption of PD-1 blockades with immunomodulatory drugs to treat multiple myeloma, but our results highlight the potential efficacy of our newly developed anti-PD-L1 Ab as a novel therapeutic.

We found that the expression of PD-L1 in multiple myeloma cells can be detected on the surface or in the nucleus. Others have indicated that the soluble PD-L1 levels in the serum of patients are associated with disease prognosis (Wang *et al*., 2015). We found that PD-L1 expression in the nucleus is also highly correlated with disease prognosis, but this requires confirmation. Also, the standard therapy drugs for multiple myeloma, such as lenalidomide and bortezomib, are associated with PD-L1 expression on the multiple myeloma cells (Görgün *et al*., 2015). Thus, rational therapy designs with anti-PD-L1 antibodies and other treatments should consider combined regimens.

In this study, we did not observe synergistic antitumor effects of lenalidomide and PD-L1 combined treatments in the murine model of myeloma (lenalidomide did not show antitumor effects against the mouse myeloma cells). This may be due to the amino acid differences between the human and mouse cereblon (CRBN) protein; isoleucine is replaced by valine at position 391 in the murine CRBN protein. Few studies have reported marginal antitumor effects of lenalidomide in syngeneic mouse models (Vo *et al*., 2018). We adopted several murine multiple myeloma models using subcutaneous, intraperitoneal, and intravenous injections of NS-1 and MOPC-1 cells in BALB/c mice, and the results showed that the newly generated anti-PD-L1 Ab had significant antitumor effects. The combined therapy of anti-PD-L1 Ab with lenalidomide did not induce synergistic or additive effects over anti-PD-L1 Ab alone, but this does not eliminate the potential benefits of combined treatments with other multiple myeloma drugs. Novel (combined) treatment options for multiple myeloma requires further investigation.

Finally, we developed a novel anti-PD-L1 antibody and this antibody can be applied to treat multiple myeloma. The newly developed anti-PD-L1 antibody showed significant antitumor effects against multiple myeloma in mice. This antibody can activate anti-cancer immune response by blocking immunocheck point molecules, and also directly eliminate cancer cells expressing PD-L1 molecule, suggesting that this anti-PD-L1 Ab is a promising candidate to treat multiple myeloma.

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