

Immunological effects of the anti-programmed death-1 antibody on human peripheral blood mononuclear cells

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Abstract. Immune checkpoint antibody-mediated blockade has gained attention as a new cancer immunotherapy strategy. Accumulating evidence suggests that this therapy imparts a survival benefit to metastatic melanoma and non-small cell lung cancer patients. A substantial amount of data on immune checkpoint antibodies has been collected from clinical trials; however, the direct effect of the antibodies on human peripheral blood mononuclear cells (PBMCs) has not been exclusively investigated. In this study, we developed an anti-programmed death-1 (PD-1) antibody (with biosimilarity to nivolumab) and examined the effects of the antibody on PBMCs derived from cancer patients. Specifically, we investigated the effects of the anti-PD-1 antibody on proliferation, cytokine production, cytotoxic T lymphocytes (CTL) and regulatory T cells. These investigations yielded several important results. First, the anti-PD-1 antibody had no obvious effect on resting PBMCs; however, high levels of the anti-PD-1 antibody partly stimulated PBMC proliferation when accompanied by an anti-CD3 antibody. Second, the anti-PD-1 antibody restored the growth inhibition of anti-CD3 Ab-stimulated PBMCs mediated by PD-L1. Third, the anti-PD-1 antibody exhibited a moderate inhibitory effect on the induction of myeloid-derived suppressor cells (MDSCs) by anti-CD3 antibody stimulation. Additionally, the presence of the anti-PD-1 antibody promoted antigen-specific CTL induction, which suggests that combining anti-PD-1 antibody and conventional immuno-

therapy treatments may have beneficial effects. These results indicate that specific cellular immunological mechanisms are partly responsible for the antitumor effect exhibited by the anti-PD-1 antibody against advanced cancers in clinical trials.

Introduction

In recent years, a novel type of cancer immunotherapy has arisen that involves a specific monoclonal antibody (mAb) targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and the programmed death-1 (PD-1) receptor/PD-ligand 1 (PD-L1) interaction. This type of therapy has been applied in many clinical trials (1-5). The mechanisms responsible for the antitumor activity of immune checkpoint blockade are thought to involve a restoration of T cell suppression mediated by tumors (6-8). The PD-1 and CTLA-4 pathways have different roles in regulating T cell activity; CTLA-4 is involved in the priming T-cell phase by interacting with antigen-presenting cells (APC) expressing co-stimulatory molecules and PD-1 is involved in the effector T cell phase by blocking cancer cells expressing PD-L1 (9).

Ipilimumab is an anti-CTLA-4 mAb, and nivolumab is an anti-PD-1 mAb; these antibodies were approved by the FDA in 2011 and 2014, respectively, for treating metastatic and unresectable melanomas. The first phase I clinical trial for nivolumab involved 296 patients and reported that the objective response rates was 17, 32 and 29% for advanced non-small cell lung cancers (NSCLC), melanoma and renal cell carcinoma (RCC), respectively, all of which had been treated heavily prior to the study (3). Additionally, nivolumab demonstrated an overall survival improvement over dacarbazine in a phase III study on previously untreated metastatic melanoma patients (10).

Despite the clinical trial success with nivolumab against advanced cancers, few preclinical studies have focused on PD-1 mAb. There are several reasons for this lack of preclinical studies: i) mouse systems cannot accurately represent the human immune response induced by the anti-PD-1 mAb, and ii) few efficient mouse anti-PD-1 antibodies are available. Currently, only a few *in vivo* studies have been published that used mouse tumor cells and mouse anti-PD-1 antibodies and that verified the antitumor response and

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Abbreviations: CTL, cytotoxic T lymphocytes; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; PBMC, peripheral blood mononuclear cell; PD-1, programmed death-1; Treg, regulatory T cells

Key words: checkpoint antibody, anti-PD-1 antibody, human peripheral blood mononuclear cell, regulatory T cells, myeloid-derived suppressor cell

specific CTL induction (11,12). However, there are difficulties associated with replacing the mouse immune system with a human system-like and then predicting the clinical response in humans from those mouse *in vivo* studies. In the future, the antitumor effect of the anti-PD-1 antibody may be investigated in a humanized mouse system that uses human immune cells and tumor cells.

In this study, we manufactured an in-house anti-PD-1 mAb that is similar to nivolumab, and we investigated the immunological effect of the antibody on the peripheral blood mononuclear cells (PBMCs) of cancer patients. We obtained specific *in vitro* immunological data by treating PBMCs with anti-PD-1 mAb. These results may contribute to the profiling of patients to predict which patients are likely to respond to anti-PD-1 mAb.

Materials and methods

Cancer patient-derived PBMCs. PBMCs from malignant glioma patients were used for *in vitro* experiments. The studies involving PBMCs derived from glioma patients were approved by the Institutional Review Board of Shizuoka Cancer Center, Shizuoka, Japan. All patients gave written informed consent. PBMCs from 6 glioma patients were used for *in vitro* cell-based assay (Table I).

Antibodies and reagents. The following antibodies were used for flow cytometric analyses: anti-CD3-PerCP, anti-CD4-PE, anti-CD8-FITC, anti-CD11b-PE-Cy7, anti-CD14-PE, anti-CD19-FITC, anti-CD25-FITC, anti-CD33-FITC, anti-CD45RO-PE, anti-CD56-biotin, anti-CCR7-biotin, anti-FoxP3-PE, anti-PD-1-APC, anti-PD-L1-APC and anti-human IFN- γ -PE. Anti-PD-1-APC and anti-PD-L1-APC antibodies were purchased from BioLegend Inc. (San Diego, CA, USA). All other antibodies were purchased from BD Pharmingen (San Diego, CA, USA). No azide/low endotoxin (NA/LE) anti-CD3 mAb was also purchased from BD Pharmingen and used for *in vitro* stimulation of human PBMCs. The WST-1 assay reagent was purchased from Dojin Kagaku Corp. (Kumamoto, Japan) and was used for cell proliferation assay. Human recombinant PD-L1 and PD-1 proteins were purchased from Sino Biotechnology (BDA, Beijing, China), and were used for a blocking assay with the anti-PD-1 mAb and for surface plasmon resonance (SPR) analysis, respectively. Commercially available unconjugated anti-PD-1 mAb was purchased from BioLegend Inc. and used for SPR assay.

Production and purification of the in-house full-length anti-PD-1 monoclonal antibody. The amino acid sequence of nivolumab was downloaded from the J-PlatPat data base from National Center for Industrial Property Information and Training (INPIT) (https://www.j-platpat.inpit.go.jp/web/toku-jitsu/tkbs/TKBS_GM401_ToPDF.action). Nivolumab-derived VH and VL genes were synthesized according to their cDNA sequences and were cloned into a pcDNA3.3 vector for IgH and IgL co-expressions. Specifically, the VH gene was ligated with human IgG4 fragment that was PCR-cloned from the human PBMC-derived cDNA, and the product was finally inserted into pcDNA3.3. These IgH and IgL vectors were expanded, purified by endotoxin-depletion and co-transduced into

Table I. The characteristics of high-grade glioma patients.

Patient code	Age	Gender	HLA-typing	Pathology
GB-001	45	M	A2	GBM (IV) ^a
GB-002	37	M	A2	AA (III)
GB-003	53	M	A24	GBM (IV)
GB-004	63	F	A2	GBM (IV)
GB-005	56	M	A24	GB (IV)
GB-006	71	M	A2	GBM (IV)

GBM, glioblastoma multiforme; AA, anaplastic astrocytoma. ^aWHO pathological grade.

expi293 cells using lipofection according to the manufacturer's instructions. The supernatant was harvested and affinity-purified with a protein A prepacked column (GE Healthcare). Finally anti-PD-1 mAb was purified as a biosimilar antibody to nivolumab and was used for *in vitro* experiments.

Surface plasmon resonance (SPR) analysis using an in-house full-length anti-PD-1 monoclonal antibody. SPR analysis was performed on a Biacore X100 (GE Healthcare) as reported previously (15). All reagents and sensor chips were purchased from GE Healthcare. Immobilization of anti-human IgG antibody was performed at pH 5.0 on the CM5 sensor chip for capturing anti-PD-1 antibody as ligand, and the amount targeted was 1,000 response units (RU). The analyte was a recombinant human PD-1 protein. Commercially available anti-PD-1 mAb was purchased from BioLegend (clone EH12.2H7) and monitored as a control.

Cell proliferation assay. Cell proliferation was examined using the WST-1 assay (Dojin Kagaku Corp., Kumamoto, Japan). Briefly, $1-2 \times 10^5$ PBMCs were seeded into each well of a 96-well micro-culture plate coated with anti-CD3 mAb at $5 \mu\text{g/ml}$ overnight at 4°C . Anti-PD-1 mAb was added at various concentrations and cells were cultured for 5 days. The WST-1 substrate was added to the culture, and the optical density (OD) was measured at 450 and 620 nm using an immunoreader (Immuno Mini NJ-2300, Nalge Nunc International, Roskilde, Denmark).

For the PD-L1 blocking assay, the anti-PD-1 mAb and the PD-L1 recombinant protein were sequentially added to a 96-well plate experiment. After a 5-day culture, the cell proliferation assay was performed using a WST-1 reagent.

Human PBMC cultures stimulated with anti-CD3 for FACS analysis. A 6-well culture plate was coated with NA/LE anti-CD3 mAb at $5 \mu\text{g/ml}$ at 4°C overnight. Human PBMCs were seeded at 8×10^6 per well in 4 ml of RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml) and 10% fetal bovine serum (FBS, Gibco, Paisley, UK). After 1 h, anti-PD-1 mAb was added at various concentrations and cells were cultured for 5 days. Then, FACS analysis was performed to analyze the T cell markers, myeloid-derived suppressor cells (MDSCs) and IFN- γ production.

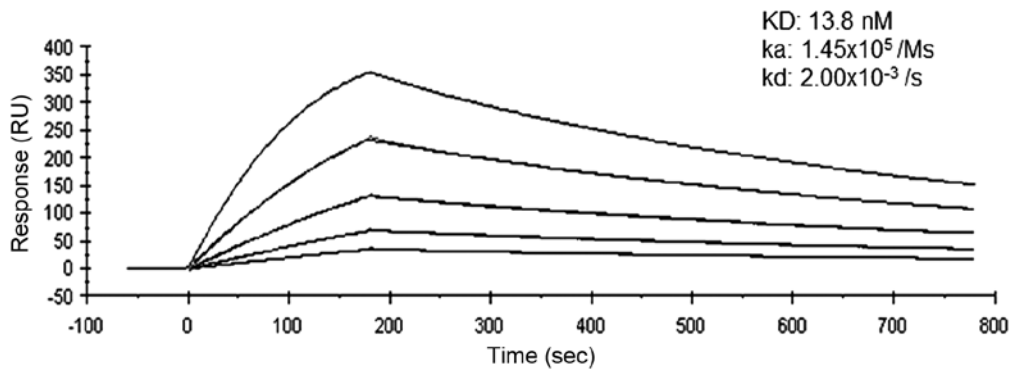


Figure 1. Determination of the affinity dynamics of a biosimilar anti-PD-1 mAb. SPR analysis of anti-PD-1 mAb binding to recombinant human PD-1 protein was performed. Binding dynamics were monitored and calculated using a Biacore X100. Anti-PD-1 mAb purchased from BioLegend (clone EH12.2H7) was monitored as a control.

For the PD-L1 blocking assay, 30 min after the addition of anti-PD-1 mAb, PD-L1 recombinant protein was added for a final concentration of 10 $\mu\text{g/ml}$ in a 6-well culture plate and cells were cultured for 5 days. For MDSC induction analysis, CD33⁺CD11b⁺ MDSC and CD14⁺CD11b⁺ monocyte MDSC fractions were measured using a flow cytometry. In the case of IFN- γ production analysis, after 5-day cultures with or without anti-PD-1 mAb and PD-L1, cells were stimulated with PMA (Sigma-Aldrich Corp., St. Louis, MO, USA) and ionomycin (Sigma) for 4 h. Finally, FACS analysis was performed to measure IFN- γ ⁺ T cells using intracellular staining with anti-human IFN- γ -PE antibody.

Propidium iodide (PI) was used for detecting living cells. Cell suspensions were harvested from cultured PBMCs and were stained with various primary antibodies for 15 min at 4°C and then washed with cold PBS+2% fetal bovine serum (FBS; Life Technologies). Then, cells were stained with the secondary antibodies for 15 min at 4°C. After washing, cells were fixed with 0.5% paraformaldehyde-containing PBS (-) and analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA).

Peptide-pulsed dendritic cell (DC)-based CTL induction assay using tetramer staining. CTL induction cultures were described previously (13). Immature DCs were induced by GM-CSF and IL-4, and mature type-1 DCs were induced by a combination of cytokines, as reported previously (14). HLA-A2-positive PBMCs derived from four glioma patients were used for CTL induction assay. Mature DCs were pulsed with HLA-A2-restricted MART-1, GP100 or CMVpp65 peptide and used for CTL induction cultures. An isotype control antibody or anti-PD-1 mAb was added to CTL cultures at a concentration of 10 $\mu\text{g/ml}$. Two-rounds of co-culture of T cells and DCs were performed, and CD8⁺tetramer⁺ cells fraction was measured using flow cytometry. More than 0.1% of CD8⁺tetramer⁺ cell frequency was rated as positive after treatment with anti-PD-1 Ab. The tetramers for MART-1, GP100 and CMVpp65 antigens were HLA-A2 restricted, and HIV-tetramer was HLA-A24 restricted.

Regulatory T cell induction assay. During two-rounds of co-culture, T cells and mature DCs were treated with or

without anti-PD-1 mAb at a concentration of 10 $\mu\text{g/ml}$ in 6-well culture plate, cells were collected and used also for Treg induction analysis. CD4⁺CD25⁺ fraction was gated and FoxP3⁺ cells were measured using flow cytometry.

Statistical analysis. The significance of differences was analyzed using Student's t-test. Values of $P < 0.05$ were considered statistically significant.

Results

Binding affinity of anti-PD-1 mAb to recombinant PD-1. SPR analysis confirmed the affinity of anti-PD-1 mAb to recombinant PD-1 consistently and mean dissociation constants were determined to be 13.8 nM (Fig. 1). The binding affinity of anti-PD-1 mAb was greater than that of the commercially available anti-PD-1 antibody (98.9 nM), as judged by SPR analysis (data not shown).

Effect of anti-PD-1 mAb on PBMC proliferation stimulated by the anti-CD3 antibody. Anti-PD-1 mAb showed no stimulatory activity on resting human PBMCs. However, high concentration of anti-PD-1 mAb exhibited moderate stimulatory effect on CD3 antibody-stimulated PBMCs from 2 of 6 patients (Fig. 2).

Effect of anti-PD-1 mAb on T cell subsets in anti-CD3 antibody-stimulated PBMC. The anti-PD-1 mAb showed no significant effect on the percentage (%) of CD3⁺CD4⁺ and CD3⁺CD8⁺ subpopulations in anti-CD3 Ab-stimulated PBMCs. Additionally, the percentage (%) of effector memory T cell subsets such as CD4⁺CD45RO⁺CD95⁺CCR7⁻ were not affected by anti-PD-1 mAb (Table II).

Effect of PD-L1-inhibited PBMC proliferation and IFN- γ production by anti-PD-1 mAb. PD-L1 at 10 $\mu\text{g/ml}$ significantly suppressed the anti-CD3 Ab-stimulated PBMC proliferation (Fig. 3). The addition of anti-PD-1 mAb at concentration >10 $\mu\text{g/ml}$ restored the growth inhibition, and interestingly at 20 $\mu\text{g/ml}$ anti-PD-1 mAb surpassed the control growth level (in the absence of PD-L1). On the other hand, IFN- γ production measured by intracellular staining showed a tendency of

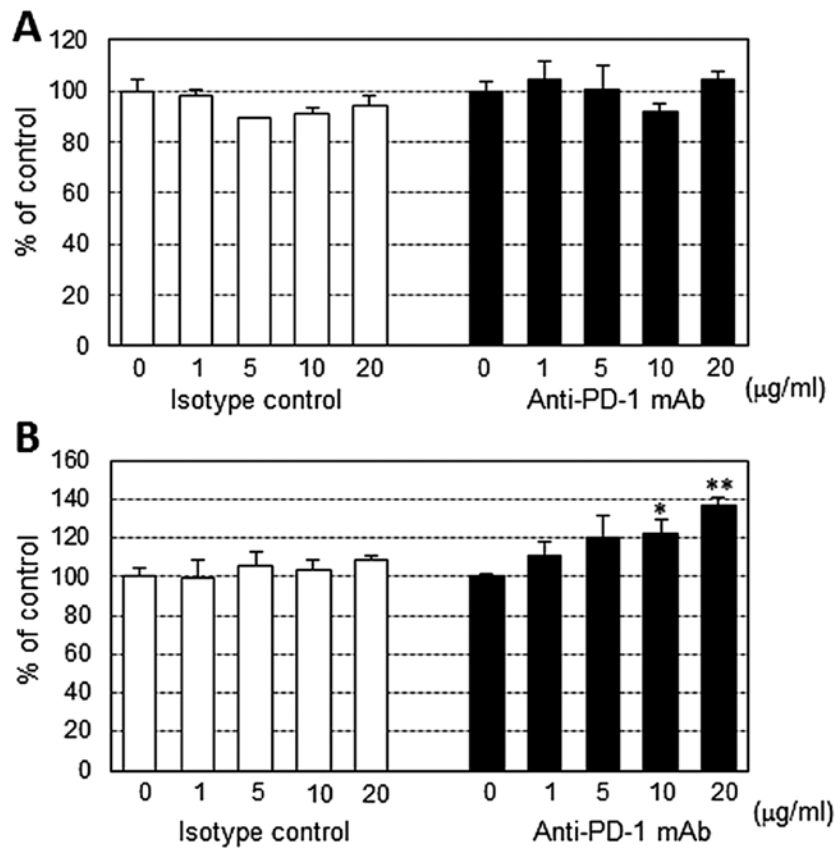


Figure 2. Effect of the anti-PD-1 mAb on anti-CD3 Ab-stimulated PBMC proliferation. Effect of anti-PD-1 mAb on (A) resting PBMCs and (B) anti-CD3 Ab-stimulated PBMCs was analyzed. Control or anti-PD-1 mAb was used at concentrations ranging from 1 to 20 µg/ml. Each graph shows a representative case from 6 sets of glioma patient-derived PBMCs, and each column indicates the mean ± SD of triplicate tests. *P<0.05, **P<0.01, statistically significant.

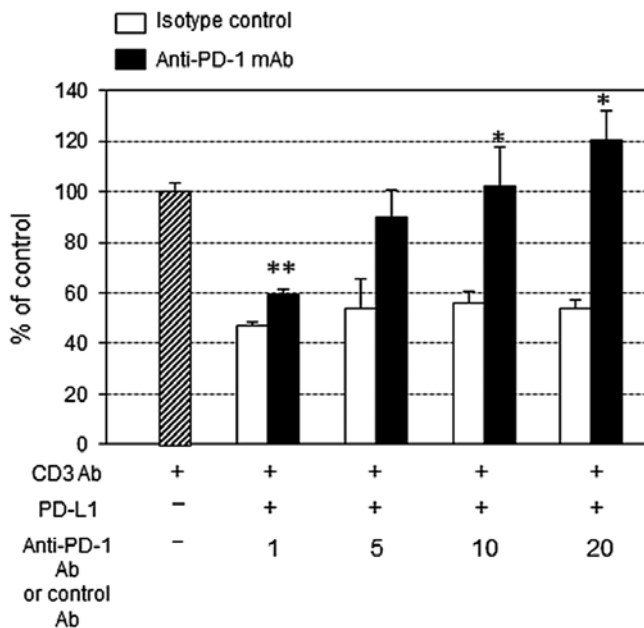


Figure 3. Blocking activity of anti-PD-1 mAb against soluble PD-L1-mediated T cell growth-inhibition. The recombinant soluble PD-L1 protein was used at 10 µg/ml and anti-PD-1 mAb was used at concentrations ranging from 1 to 20 µg/ml. The graph shows a representative case from 6 sets of glioma patient-derived PBMCs, four of which demonstrated a recovery from PD-L1-induced T cell inhibition. Each column indicates the mean ± SD of triplicate tests. Open column, isotype control Ab; closed column, anti-PD-1 mAb; shaded column, no PD-L1 and no anti-PD-1 mAb. *P<0.05, **P<0.01, statistically significant.

restoration in anti-PD-1 mAb compared to isotype control cultures containing PD-L1, however it was not statistically significant (Fig. 4).

Effect of anti-PD-1 mAb on MDSC induction. A small number of CD33⁺CD11b⁺ MDSCs and CD14⁺CD11b⁺ monocyte MDSCs were identified in anti-CD3 antibody-stimulated PBMC cultures. The addition of anti-PD-1 mAb inhibited monocyte MDSC induction by ~40% compared to the control (Fig. 5A and B). PD-L1 expression was observed in 60% of CD33⁺CD11b⁺ MDSCs; however, anti-PD-1 mAb did not show significant effects on PD-L1 expression (Fig. 5C).

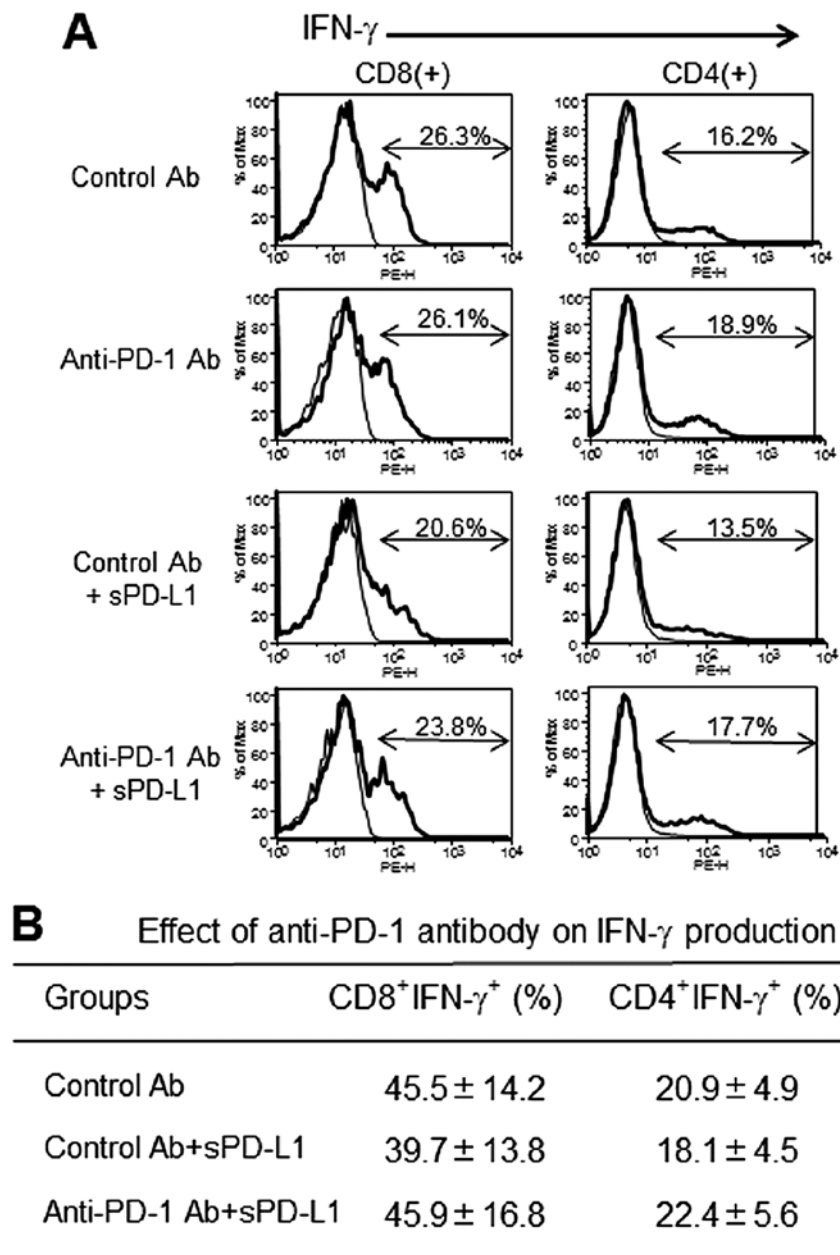
Stimulatory effect of anti-PD-1 mAb on antigen-specific CTL induction. Antigen peptide (MRAT-1, GP100, CMVpp65)-specific CTLs with HLA-A2 restriction significantly increased in anti-PD-1 Ab-treated CTL cultures compared to control Ab-treated cultures (Fig. 6A and B and Table III). In contrast, no CTLs responded to HLA-A24-restricted HIV peptide. Interestingly, mature DCs demonstrated higher PD-L1 expression than immature DCs (Fig. 6D).

Effect of anti-PD-1 mAb on Treg induction. Regulatory T cell induction was investigated after two-rounds of CMVpp65 peptide-pulsed DC-mediated CTL stimulation. The frequency of CD4⁺CD25⁺ fraction was not different between control and anti-PD-1 Ab treated cultures. However, the frequency of

Table II. Effect of anti-PD-1 antibody on T cell subsets in PBMCs.

Anti-PD-1 Ab ($\mu\text{g/ml}$)	T cell marker (%)			Memory T cell marker (%)
	CD3 ⁺	CD3 ⁺ CD4 ⁺	CD3 ⁺ CD8 ⁺	CD4 ⁺ CD45RO ⁺ CD95 ⁺ CCR7 ^{-a}
0	80.4 \pm 6.5	59.7 \pm 20.3	24.1 \pm 17.1	17.1 \pm 2.8
10	83.4 \pm 7.5	60.9 \pm 16.4	26.8 \pm 10.2	20.8 \pm 1.5
20	82.3 \pm 3.3	64.7 \pm 18.2	27.1 \pm 14.9	23.3 \pm 3.1

^aCD4⁺ fraction was gated and specific memory T cell subset was measured. Each value shows mean \pm SD of PBMC data derived from 6 glioma patients.



CD4⁺ or CD8⁺ T cell fraction was gated and IFN- γ ⁺ cells were measured. Each value shows mean \pm SD of PBMC data derived from 6 glioma patients.

Figure 4. Effects of anti-PD-1 mAb on PD-L1-mediated inhibition of IFN- γ production. Recombinant soluble PD-L1 protein was used at 10 $\mu\text{g/ml}$, and the isotype control and anti-PD-1 mAb were used at 10 $\mu\text{g/ml}$. (A) Flow cytometry analysis of IFN- γ ⁺ T cells. The histogram shows a representative case from 6 sets of glioma patient-derived PBMCs. Thin line, PE-labeled control antibody; thick line, PE-labeled anti-IFN- γ antibody. (B) The frequency of IFN- γ ⁺ CD4⁺ or CD8⁺ T⁺ cells in the presence of PD-L1 with or without anti-PD-1 mAb.

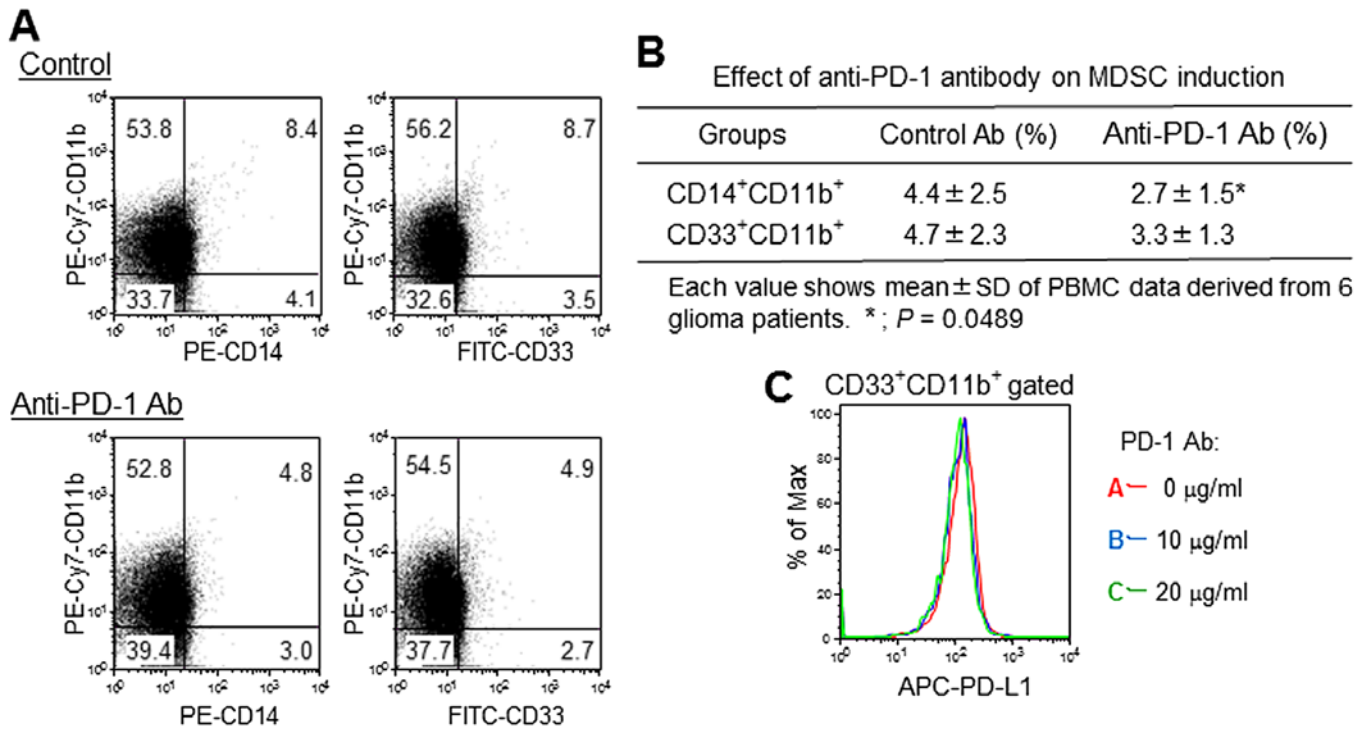


Figure 5. Inhibitory effect of anti-PD-1 mAb on MDSC induction in anti-CD3 Ab-stimulated PBMCs. (A) Reduction in CD33⁺CD11b⁺ MDSC and CD14⁺CD11b⁺ monocyte MDSC numbers by anti-PD-1 mAb. The flow cytometry data show a representative case from 6 sets of glioma patient-derived PBMCs. Each value indicates the frequency in %. (B) The frequency of CD33⁺CD11b⁺ and CD14⁺CD11b⁺ MDSCs with or without anti-PD-1 mAb. (C) PD-L1 expression in CD33⁺CD11b⁺ MDSC from PBMCs treated with various concentrations of anti-PD-1 mAb.

Table III. Effect of anti-PD-1 Ab on tumor antigen-specific CTL inductions.

Patient/ tetramer	CD8 ⁺ tetramer ⁺ cell (%)			
	MART-1	GP100	CMVpp65	A2 HIV A24
GB-001	0.02 ^b /0.39 ^{a,c}	0.16/0.26 ^a	0.01/0.01	0/0.01
GB-002	0.01/0.01	0.02/0.74 ^a	0.02/0.11 ^a	0/0.02
GB-004	0.02/0.12 ^a	0.15/0.27 ^a	0.09/0.17 ^a	0/0.02
GB-006	0.04/0.05	0.05/0.06	0.38/1.13 ^a	ND

Each value shows CD8⁺tetramer⁺ cell frequency. ^aMore than 0.1% of CD8⁺tetramer⁺ cell frequency was rated as positive after treatment with anti-PD-1 Ab. ^bTreated with control Ab. ^cTreated with anti-PD-1 Ab. ND, not done.

FoxP3⁺ cells in gated CD4⁺CD25⁺ fraction was inhibited in anti-PD-1 Ab-treated cultures (Fig. 6C).

Discussion

Since the development of anti-CTLA-4 Ab, ipilimumab has been administered to metastatic melanoma patients as an anti-check-point Ab (1,16). More immunomodulatory Abs such as anti-PD-1 (3,17), anti-PD-L1 (4), anti-CD137 (18) and anti-CD40 (19,20) have been developed and may be applicable

to various advanced cancers. Ipilimumab treatment resulted in >20% responders; in addition, the antibody resulted in long-term survival in metastatic melanoma patients despite adverse effects (1,2). Remarkably, combination therapy with ipilimumab and nivolumab has shown great success in phase III clinical trials, with >50% response rate (21,22).

Intensive search for suitable biomarkers has been performed to enable responder prediction prior to treatment. This search uncovered blood biomarkers such as an increase in lymphocyte number, a decrease in LDH and MDSC numbers, and intratumoral biomarkers such as an increase in infiltrating CD8⁺ T cell numbers and granzyme and a decrease in regulatory T cell numbers (23,24).

Similarly to ipilimumab, nivolumab has been reported to show a remarkable antitumor response and survival benefit in advanced melanoma and non-small cell lung cancer patients; however, the antibody was also found to induce autoimmune effects in thyroid and lung cancer patients in a phase III clinical trial (22,25,26). With regard to biomarkers, genetic and cytokine markers have been intensively investigated using T cells and monocytes derived from cancer patients who have been treated with a combination of ipilimumab and nivolumab. These investigations demonstrated an increase in T cell proliferation, as well as upregulation of chemokine and NK cell function-associated genes (27).

Clinical trial studies demonstrated that a heterogeneous response to antibody therapy is expected for individuals, and the prediction of response will be difficult in spite of strong biomarkers. For this reason, the direct observation of the PBMC response to Ab treatment on an individual basis would

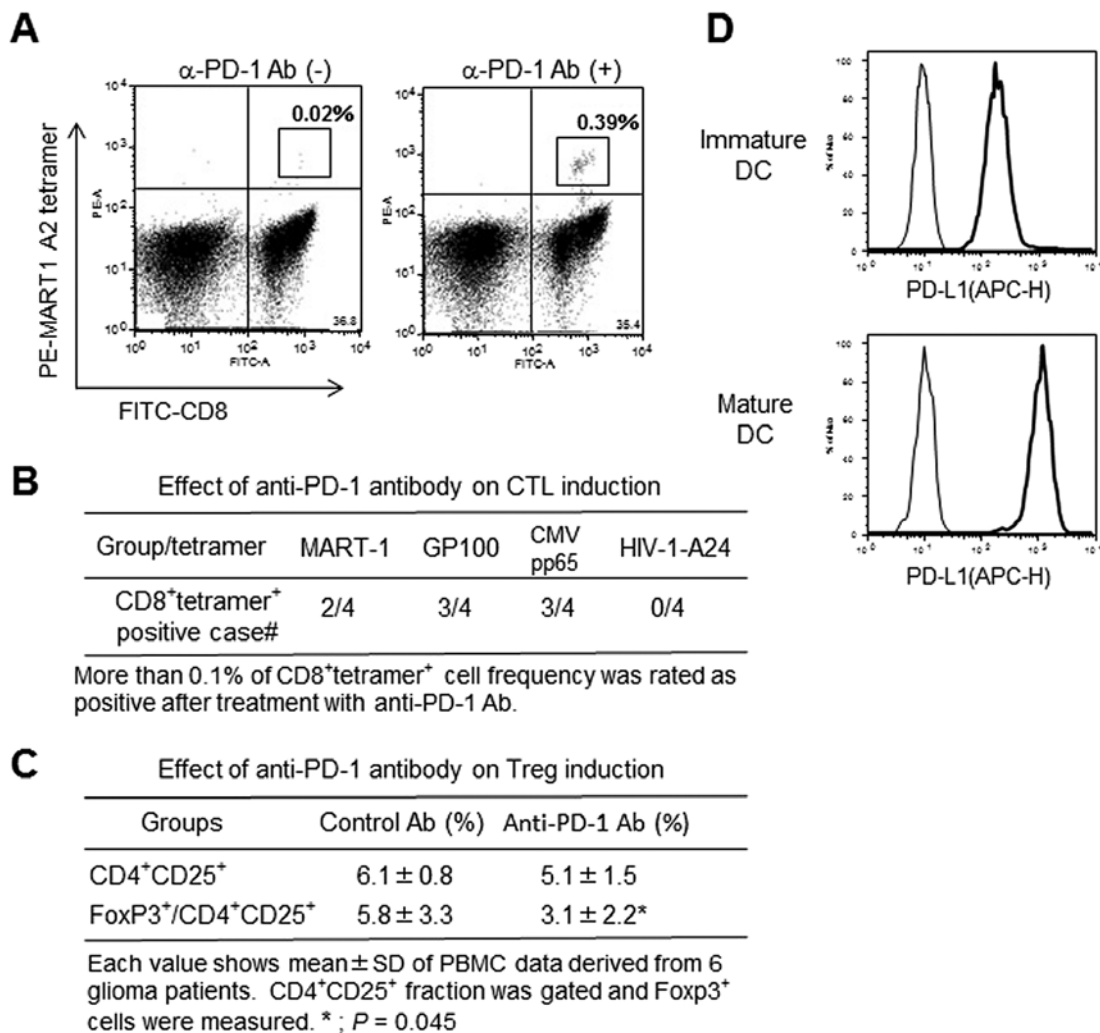


Figure 6. Effect of anti-PD-1 mAb on tumor antigen-specific CTL and Treg induction. (A) CD8⁺MART-1 A2-tetramer⁺ CTLs induction using mature DCs. Mature type-1 DCs were induced by a combination of cytokines such as TNF α , IL-1 β , IFN- α , IFN- γ plus poly I/C. Isotype control antibody or anti-PD-1 mAb was added to CTL cultures at 10 μ g/ml. Flow cytometry data indicate a representative case from 4 sets of glioma patient-derived PBMCs. (B) CD8⁺A2-tetramer⁺ CTL-positive case numbers of total 4 cases are shown. The tetramers for MART-1, GP100 and CMVpp65 were HLA-A2-restricted, and HIV-1 tetramer was HLA-A24 restricted. (C) The frequencies of CD4⁺CD25⁺ and FoxP3⁺ of CD4⁺CD25⁺ with or without anti-PD-1 mAb are shown. (D) PD-L1 expression on immature or mature DCs. Thin line, isotype control antibody; thick line, anti-PD-L1 mAb.

be helpful for predicting the immunological response for the patients in question. A substantial amount of evidence from many clinical trials has accumulated on this subject. However, *in vitro* research using nivolumab has not been extensively performed. In this study, we manufactured a biosimilar mAb to nivolumab in-house and evaluated its biological function using specific immunological assays. SPR analysis showed that our anti-PD-1 antibody had a K_D value of 13.8 nM, and that of nivolumab is 3.06 nM (28). Our in-house anti-PD-1 antibody seems to have a greater affinity than other commercially available anti-PD-1 monoclonal antibodies (98.9 nM, data not shown).

Wang *et al* (28) demonstrated that nivolumab showed stimulatory activity in *in vitro* experiments, such as in MLR assays and cytokine production experiments; three positive observations were verified: i) T cell growth was stimulated and IFN- γ production increased in co-culture with allogeneic dendritic cells (DCs), ii) regulatory T cell-mediated T cell growth inhibition was reversed, and iii) a synergistic

increase in specific antibody titer after antigen vaccination in non-human primate resulted. However, no *in vitro* antibody-dependent cell cytotoxicity was detected when nivolumab was used against activated T cells.

In this study, our anti-PD-1 antibody stimulated the proliferation of T cells activated with anti-CD3 antibody even at high antibody concentration in only a few cases (20 μ g/ml). In particular, anti-PD-1 antibody restored the PD-L1-mediated T cell growth inhibition. These observations are consistent with those in previous studies.

Regarding the MDSC induction by anti-CD3 antibody stimulation, anti-PD-1 antibody inhibited MDSC induction in response to anti-CD3 antibody-stimulated PBMCs. The MDSC populations, CD33⁺CD11b⁺ and CD14⁺CD11b⁺, are reported to be induced in the peripheral blood of cancer patients treated with chemotherapy (29,30). The inhibitory effect shown by the anti-PD-1 antibody on MDSC induction represents the first report in the study of the immunological function of the anti-PD-1 antibody using human PBMCs.

In the near future, the effect of anti-PD-1 antibody on the MDSC inhibitory action on immune cells should be investigated to clarify the mechanism of antitumor effect of anti-PD-1 antibody.

Similarly, regulatory T cell induction mediated by mature DCs was suppressed by the addition of anti-PD-1 antibody in CTL induction cultures. Wang *et al*, Klein *et al* and Wong *et al* emphasized the restorative activity of the anti-PD-1 antibody on the regulatory T cell-mediated inhibition of effector T cell activation and cytokine production (7,28,31,32). These observations that the anti-PD-1 antibody reversed the immunological inhibition by regulatory T cells and MDSCs *in vitro*, suggest that the anti-PD-1 antibody induces antitumor activity by restoring the immunosuppressive state and by activating T cell function.

Importantly, DC-mediated antigen-specific CTL induction was potentiated more efficiently in the presence of the anti-PD-1 antibody. Therefore, this observation suggests that it may be beneficial to develop a combined anti-PD-1 antibody and DC vaccine as a therapy (33,34).

Finally, to develop an anti-PD-1 antibody therapy model that is more significant than *in vitro* studies, we have developed an autologous immunotherapy *in vivo* model based on humanized NOG mice (35), in which both the autologous immune system and autologous tumors can be established. This autologous immunotherapy *in vivo* model will next be used to predict the immunological effect in cancer patients who will receive anti-PD-1 antibody therapy.

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