学 会 等 発 表 実 績

委託業務題目「オリジナル抗原HSP105由来ペプチドワクチンのFIH医師主導治験」

機関名　独立行政法人国立がん研究センター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Glypican-3(GPC3) ペプチドワクチン投与後の投与局所及び腫瘍局所でのペプチド特異的CTLの解析（ポスター発表）	吉川聡明 下村真菜美 澤田雄 高橋真理 吉原宏樹 上野浩生 真部淳 細野亜古 植村靖史 中面哲也	第18回日本がん免疫学会総会（松山）	2014年7月30日～8月1日	国内
Glypican-3由来エピトープペプチド結合リポソームのCTL誘導能の評価（ポスター発表）	岩間達章 内田哲也 下村真菜美 吉川聡明 中面哲也	第18回日本がん免疫学会総会（松山）	2014年7月30日～8月1日	国内
Analysis of glypican-3 specific CTLs in the tumor tissue and vaccination site after administration of GPC3 peptide.（Glypican-3(GPC3) ペプチドワクチン投与後の投与局所及び腫瘍局所でのペプチド特異的CTLの解析）（ポスター発表）	吉川聡明 下村真菜美 澤田雄 植村靖史 中面哲也	第73回日本癌学会学術総会（横浜）	2014年9月25日～27日	国内
Evaluation of peptide-specific CTL-inducible ability of glypican-3-derived p eptide-coupled liposome vaccine.（Glypican-3由来ペプチドを結合したリポソームワクチンのペプチド特異的CTL誘導能評価）（ポスター発表）	岩間達章 内田哲也 下村真菜美 吉川聡明 中面哲也	第73回日本癌学会学術総会（横浜）	2014年9月25日～27日	国内
The enhancement of the CTL induction by peptide vaccine therapyin combinatio n with anti-CD4 antibody.（抗CD4抗体の併用投与は抗腫瘍ペプチドワクチン療法のCTLプライミング効率を高める）（ポスター発表）	藤浪紀洋 吉川聡明 澤田雄 下村真菜美 岩間達章 植村靖史 中面哲也	第73回日本癌学会学術総会（横浜）	2014年9月25日～27日	国内

EGFR T790M mutation-derived antigen provides the immunogenicity in NSCLC patients. （非小細胞肺がんにおけるEGFR T790M変異由来抗原は免疫原性を与える） （ポスター発表）	大藤和也 吉川聡明 多田好孝 吉村麻友子 下村真菜美 中本安成 中面哲也	第73回日本癌学会学術総会 （横浜）	2014年9月25日～27日	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所 （学会誌・雑誌等名）	発表した時期	国内・外の別
A peptide antigen derived from EGFR T790M is immunogenic in non-small cell lung cancer.	Ofuji K. Tada Y. Yoshikawa T. Shimomura M. Yoshimura M. Saito K. Nakamoto Y. Nakatsura T.	Int. J. Oncol.	2015	国外
Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes.	Sawada Y. Yoshikawa T. Shimomura M. Iwama T. Endo I. Nakatsura T.	Int. J. Oncol.	2015	国外
Identification of HLA-A2 or HLA-A24-restricted CTL epitopes for potential HSP105-targeted immunotherapy in colorectal cancer.	Sawada Y. Komori H. Tsunoda Y. Shimomura M. Takahashi M. Baba H. Ito M. Saito N. Kuwano H. Endo I. Nishimura Y. Nakatsura T.	Oncol. Rep.	2014	国外
Clinical development of immune checkpoint inhibitors.	Ito A. Kondo S. Tada K. Kitano S.	BioMed Research International.	1015	国外
Computational Algorithm Driven Evaluation of Monocytic Myeloid Derived Suppressor Cell Frequency For Prediction of Clinical Outcomes.	Kitano S. Postow MA. Ziegler CG. Kuk D. Panageas K. Cortez C. Rasalan TS. Adamow M. Yuan J. Wong P. Altan-Bonnet G. Wolchok JD. Lesokhin AM.	Cancer Immunol Res.	2014	国外

抗PD-1抗体の臨床試験の現状	北野滋久 藤原豊	がん分子標的治療	2014	国内
悪性黒色腫に対する抗PD-1抗体療法：抗PD-1抗体と抗CTLA-4抗体併用療法	北野滋久	細胞工学	2014	国内
Tregによる免疫反応の制御とimmune checkpointによる免疫修飾の違い	北野滋久 塚崎邦弘	腫瘍内科	2014	国内
記録 第3回 がん新薬開発合同シンポジウム 研究者主導未承認薬開発試験の実施および規制上の諸問題－アカデミアの立場から－	佐藤暁洋	腫瘍内科	2014	国内

Ⅴ．研究成果の刊行物・別刷

A peptide antigen derived from EGFR T790M is immunogenic in non-small cell lung cancer

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Received August 29, 2014; Accepted October 9, 2014

DOI: 10.3892/ijo.2014.2787

Abstract. Lung cancer is the leading cause of cancer-related deaths worldwide. Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, have demonstrated marked clinical activity against non-small cell lung cancer (NSCLC) harboring activating epidermal growth factor receptor (EGFR) mutations. However, in most cases, patients develop acquired resistance to EGFR-TKI therapy. The threonine to methionine change at codon 790 of EGFR (EGFR T790M) mutation is the most common acquired resistance mutation, and is present in ~50% cases of TKI resistance. New treatment strategies for NSCLC patients harboring the EGFR T790M mutation are required. We evaluated the immunogenicity of an antigen derived from EGFR with the T790M mutation. Using BIMAS we selected several EGFR T790M-derived peptides bound to human leukocyte antigen (HLA)-A*02:01. T790M-A peptide (789-797) (IMQLMPFGC)-specific cytotoxic T lymphocytes (CTLs) were induced from peripheral blood mononuclear cells (PBMCs) of HLA-A2$^+$ healthy donors. An established T790M-A-specific CTL line showed reactivity against the NCSLC cell line, H1975-A2 (HLA-A2$^+$, T790M$^+$), but not H1975 (HLA-A2$^-$, T790M$^+$), and the corresponding wild-type peptide (ITQLMPFGC)-pulsed T2 cells using an interferon-γ (IFN-γ) enzyme-linked immuno spot (ELISPOT) assay. This CTL line also demonstrated peptide-specific cytotoxicity against H1975-A2 cells. This finding suggests that the EGFR T790M mutation-derived antigen could be a new target for cancer immunotherapy.

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Abbreviations: aAPC, artificial antigen-presenting cell; ELISPOT, enzyme-linked immuno spot; HLA, human leukocyte antigen; IFN-γ, interferon-γ; MAPK, mitogen-activated protein kinase; PBMC, peripheral blood mononuclear cell; PI3K, phosphatidylinositol 3-kinase; PFS, progression-free survival; STAT, signal transducer and activator of transcription

Key words: acquired resistance, CTL epitope, EGFR T790M, immunotherapy, non-small cell lung cancer

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~80% of all lung cancer cases. Despite recent development in treatment agents, the prognosis for lung cancer patients remains poor (2).

Overexpression of epidermal growth factor receptor (EGFR) is observed in various malignancies, including lung cancer (3). EGFR activation induces many intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and signal transducer and activator of transcription (STAT) pathways, which cause tumor cell proliferation and survival (4). The EGFR pathway is an appropriate target for cancer therapy, and several agents that block this pathway have been developed. In particular, epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs), such as gefitinib and erlotinib, demonstrated marked clinical activity against NSCLC harboring an activating EGFR mutation (5-9). However, patients develop acquired resistance to EGFR-TKIs almost without exception (10). A secondary mutation, resulting in a threonine to methionine change at codon 790 of EGFR (EGFR T790M), is the major mechanism of EGFR-TKI resistance (10,11). Additionally, some reports suggest that the EGFR T790M mutation may not be rare and may exist in a small population of in tumor cells before TKI treatment (12-14). Moreover, a pre-existing T790M mutation was associated with shorter progression-free survival (PFS) in patients receiving TKI treatment (13,14). At this time, no standard treatment for EGFR mutant patients with acquired resistance has yet been established, and novel strategies for overcoming this resistance issue are required.

Immunotherapy for NSCLC patients is considered to be a potentially feasible option, because of its high specificity

and low toxicity against normal tissues; indeed, several tumor-associated antigen (TAA)-targeted phase 2/3 studies are ongoing (15). However, unfortunately, the results of a TAA-based vaccine therapy study were unsatisfactory (16). One concept for improving the effect of cancer vaccine therapy is to target mutated antigen-derived epitopes. It has been reported that various mutated epitopes were recognized by tumor-reactive T cells (17,18), suggesting that the mutated epitope was potentially immunogenic and thus might function as an immunotherapeutic target. There are few studies of immunotherapy targeting the EGFR T790M mutation. Here, we hypothesized that EGFR T790M-harboring cancer cells could be targeted by activated immune cells, and attempted to assess the immunogenicity of the EGFR T790M mutation-derived antigen *in vitro*. In the present study, we identified the human leukocyte antigen (HLA)-A2-restricted EGFR T790M mutation-derived epitope. Our results suggest that immunotherapy targeting the EGFR T790M mutation-derived antigen may be a novel treatment option for NSCLC patients with the T790M mutation. The combination of immunotherapy and EGFR-TKI therapy also may be a novel strategy for prevention of T790M-mediated resistance.

Materials and methods

Cell lines. The human NSCLC cell line H1975 was provided by Professor Seiji Yano (Kanazawa University, Ishikawa, Japan). H1975-A2 (H1975 transfected with HLA-A2) was provided by Dr Tetsuro Sasada (Kurume University, Fukuoka, Japan). Artificial APC-A2 (aAPC-A2) cells, which were generated by transduction of HLA-A*02:01, CD80, and CD83 molecules into K562 cells, were provided by Dr Naoto Hirano (Dana-Farber Cancer Institute, Boston, MA, USA). T2 cells (HLA-A*02:01, TAP^-) and human NSCLC cell line 11-18 were purchased from Riken (Saitama, Japan). These cell lines were cultured in RPMI-1640 (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% FBS (Gibco-BRL, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2.

PBMC collection. Peripheral blood samples were collected from four HLA-A*02:01-positive healthy donors, after informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen until use.

Epitope prediction and synthesis. The epitope prediction software BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) was used to predict peptides that could bind to HLA-A2. EGFR T790M mutation-derived peptides (purity >95%) were purchased from Scrum, Inc. (Tokyo, Japan). H-2 Kb-restricted ovalbumin (OVA) (257-264) (SIINFEKL) peptide (AnaSpec, Inc., Fremont, CA, USA) was used as a negative control in the peptide-binding assay. HLA-A2-restricted cytomegalovirus (CMV) (495-503) (NLVPMVATV) peptide was used as a positive control peptide, and an HLA-A2-restricted HIV-gag (77-85) (SLYNTYATL) peptide (American Peptide Company, Sunnyvale, CA, USA) as an irrelevant peptide in cytotoxic T lymphocyte (CTL) assays.

Peptide-binding assay. After incubation in culture medium at 26˚C overnight, T2 cells were washed with PBS and suspended in 1 ml Opti-MEM (Invitrogen Life Technologies, Carlsbad, CA, USA) with peptide (100 μg/ml), followed by incubation at 26˚C for 3 h and then at 37˚C for 2.5 h. After washing with PBS, HLA-A2 expression was measured using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) using a FITC-conjugated HLA-A2 (MBL Co., Ltd., Aichi, Japan)-specific monoclonal antibody. Mean fluorescence intensity (MFI) was analyzed using the FlowJo software (Tomy Digital Biology Co., Ltd., Tokyo, Japan). An OVA peptide was used as a negative control. A CMV peptide was used as a positive control peptide.

Generation of DCs. $CD14^+$ cells were isolated from PBMCs using human CD14 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Immature dendritic cells (DCs) were generated from $CD14^+$ cells using IL-4 (10 ng/ml; PeproTech, Inc., Rocky Hill, NJ, USA) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/ml; PeproTech, Inc.) in RPMI-1640 supplemented with 10% FBS. Maturation of DCs was induced by prostaglandin E2 (PGE2) (1 μg/ml; Sigma Chemical Co.) and tumor necrosis factor-α (TNF-α) (10 ng/ml; PeproTech, Inc.).

Induction of peptide-specific CTLs. $CD8^+$ cells were isolated using human CD8 microbeads (Miltenyi Biotec GmbH) from PBMCs. $CD8^+$ cells ($2x10^6$ cells/well) were stimulated with peptide-pulsed (10 μg/ml) 100-Gy-irradiated autologous mature DCs ($1x10^5$ cells/well) in RPMI-1640 containing 10% heat-inactivated human AB serum. After 1 week, these cells were stimulated twice weekly with peptide-pulsed (10 μg/ml) 200-Gy-irradiated aAPC-A2 cells ($1x10^5$ cells/well). Supplementation with 10 IU/ml IL-2 (Proleukin; Novartis, Basel, Switzerland) and 10 ng/ml IL-15 (PeproTech, Inc.) was performed at 3-4-day intervals between stimulations.

IFN-γ ELISPOT assay. Specific secretion of interferon-γ (IFN-γ) from human CTLs in response to stimulator cells was assayed using the IFN-γ enzyme-linked immuno spot (ELISPOT) kit (BD Biosciences), according to the manufacturer's instructions. Stimulator cells were pulsed with peptide for 2 h at room temperature and then washed three times. Responder cells were incubated with stimulator cells for 20 h. The resulting spots were counted using an ELIPHOTO counter (Minerva Tech, Tokyo, Japan).

CD107a assay and generation of a CTL line. $CD8^+$ cells isolated using human CD8 microbeads from cultured cells were incubated with peptide-pulsed T2 cells at a ratio of 2:1 for 3.5 h at 37˚C. CD107a-specific antibodies (BD Biosciences) were included in the mixture during the incubation period. $CD8^+$ $CD107a^+$ cells were sorted using a FACSAria II cell sorter (BD Biosciences). Sorted CTLs were stimulated, and the CTL line was established as described previously (19).

Cytotoxicity assay. Cytotoxic capacity was analyzed using the Terascan VPC system (Minerva Tech). The CTL line

Table I. Predicted EGFR T790M-derived peptides binding to HLA-A2.

Peptide name	Position	Length	Sequence	BIMAS score[a]
T790M-A	789-797	9	IMQLMPFGC	35.378
T790M-B	790-799	10	MQLMPFGCLL	51.77
T790M-C	788-797	10	LIMQLMPFGC	24.921
T790M-D	789-797[b]	9	IMQLMPFGV	495.288
T790M-E	789-797[c]	9	IMQLMPFGL	152.124
T790M-Awt	789-797	9	ITQLMPFGC	0.68

[a]Binding scores were estimated using the BIMAS software (http://www-bimas.cit.nih.gov/molbio/hla_bind/). [b,c]The cysteine (C) residue at position 797 was mutated to valine (V) and leucine (L), respectively. EGFR T790M, threonine to methionine change at codon 790 of EGFR.

was used as the effector cell type. Target cells were labeled in calcein-AM (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution for 30 min at 37°C. The labeled cells were then co-cultured with the effector cells for 4-6 h. Fluorescence intensity was measured before and after the culture period, and specific cytotoxic activity was calculated using the following formula: % cytotoxicity = {1 - [(average fluorescence of the sample wells - average fluorescence of the maximal release control wells)/(average fluorescence of the minimal release control wells - average fluorescence of the maximal release control wells)]} x 100%.

Results

*Assessment of EGFR T790M-derived peptide binding to HLA-A*02:01 molecules.* As the candidates of HLA-A*02:01-restricted EGFR T790M-derived CTL epitopes, we selected five 9- or 10-mer peptides with high predicted HLA-A*02:01-binding scores, calculated using BIMAS software. Three of the five EGFR T790M-derived peptides had higher binding scores than the corresponding wild-type peptides. Some studies have reported that modified peptides with single amino acid substitutions exhibit improved affinity for HLA molecules and enhanced immunogenicity (20-22); thus, we also designed two modified peptides. These modified peptides with a substitution of Cys for Val (T790M-D) or Leu (T790M-E) at codon 797 showed higher binding scores (Table I).

Using the HLA-A2 TAP-deficient T2 cell line, the binding affinity of the five synthetic peptides to HLA-A2 was assessed. A peptide-binding assay showed that three EGFR T790M-derived peptides were able to bind to HLA-A*02:01 molecules. In particular, the binding capability of the T790M-A peptide to HLA-A*02:01 molecules was higher than that of the corresponding wild-type peptide. This result suggests that the single amino acid substitution at codon 790 improved the binding affinity for HLA-A*02:01 molecules. The binding affinities of two mutated peptides (T790M-D and -E) to HLA-A*02:01 were equivalent to that of the CMV peptide used as a positive control (Fig. 1).

Induction of EGFR T790M-derived peptide-specific CTLs from human PBMCs. To evaluate the immunogenic potential of the five predicted HLA-A*02:01-binding peptides derived from EGFR T790M, we attempted to induce peptide-specific

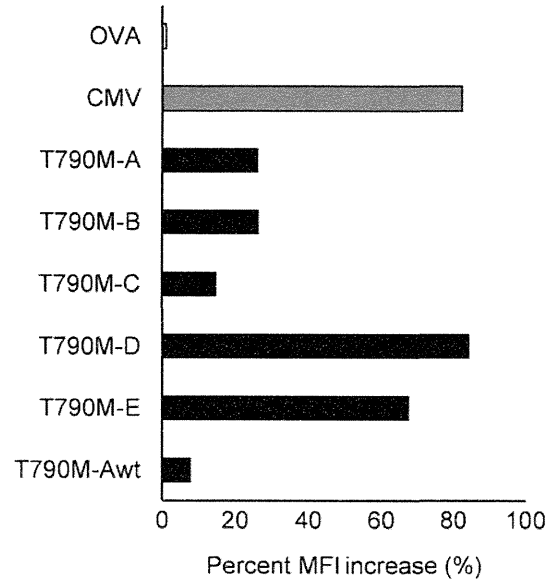

Figure 1. Binding of threonine to methionine change at codon 790 of EGFR (EGFR T790M)-derived peptides to human leukocyte antigen (HLA)-A2 molecule. A T2 binding assay was performed using a FACS system. An ovalbumin (OVA) peptide was used as a negative control. The bars show percent increases in mean fluorescence intensity (MFI). The average of two independent experiments is shown. (Percent MFI increase) = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) x 100.

CTLs from human PBMCs obtained from four healthy donors. Several reports have shown the usefulness of artificial antigen-presenting cells (aAPCs) for the induction and expansion of peptide-specific CTLs from PBMCs (23,24). Thus, we attempted to induce such CTLs using aAPCs. $CD8^+$ cells were isolated from human PBMCs using human CD8 microbeads, and then stimulated with peptide-pulsed DCs for 1 week and subsequently, stimulated twice weekly with peptide-pulsed aAPC-A2 (Fig. 2A). As shown in Fig. 2B, ELISPOT assays revealed that T790M-A (789-797) (IMQLMPFGC)-specific CTLs were induced from PBMCs from all four donors. Also, induction of T790M-B (790-799) (MQLMPFGCLL)-specific CTLs were induced from PBMCs from two of the four healthy donors. However, stimulation with three other peptides, including modified peptides, did not induce peptide-specific CTLs. These results suggest that T790M-A (789-797) and T790M-B (790-799) have immunogenic potential and that

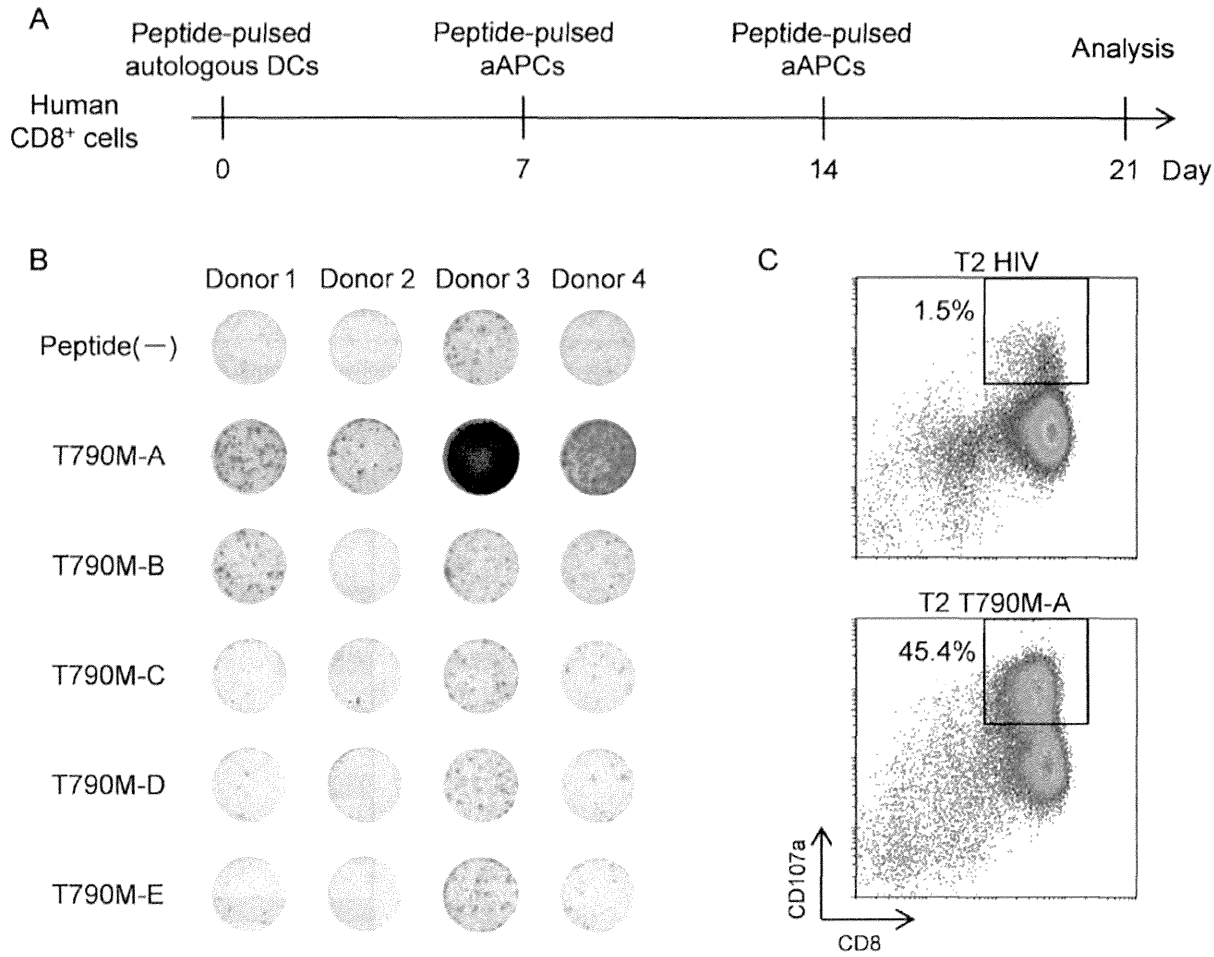

Figure 2. Induction of threonine to methionine change at codon 790 of EGFR (EGFR T790M)-derived peptide-specific cytotoxic T lymphocytes (CTLs) from peripheral blood mononuclear cells (PBMCs) of healthy donors. (A) Induction schedule of peptide-specific CTLs. $CD8^+$ cells ($2x10^6$ cells) isolated by anti-human CD8 microbeads from PBMCs were incubated with 10 μg/ml peptide-pulsed autologous dendritic cells (DCs) ($1x10^5$ cells) on day 0, followed by incubation with 10 μg/ml peptide-pulsed artificial antigen-presenting cell (aAPCs) ($1x10^5$ cells) on days 7 and 14. Peptide specificity was assessed by interferon-γ (IFN-γ) enzyme-linked immuno spot (ELISPOT) assay on day 21. (B) IFN-γ ELISPOT assay was carried out (effector, $1x10^5$ cells/well; target, $1x10^5$ cells/well) in duplicate at least three times independently; representative data are shown. (C) T790M-A-specific CTLs of healthy donor 3 were incubated with 10 μg/ml peptide-pulsed T2 cells (E:T = 2:1) for 3.5 h in the presence of an anti-human CD107a antibody. $CD8^+$ $CD107a^+$ cells were sorted using a FACSAria II cell sorter, which resulted in establishment of a T790M-A-specific CTL line.

CTLs specific for these peptides can be induced from human PBMCs. Given the effective induction of T790M-A (789-797) peptide-specific CTLs, we performed further analysis of the T790M-A peptide.

Generation of EGFR T790M-A-specific CTL line from human PBMCs. Next, we attempted to generate a purified T790M-A (789-797)-specific CTL line. Because the surface mobilization of CD107a is useful for identifying and isolating functional tumor-reactive T cells (25), we performed a CD107a assay to generate a purified T790M-A (789-797)-specific CTL line. Cultured cells stimulated by T790M-A peptide-pulsed DCs and aAPC-A2 *in vitro* were incubated with peptide-pulsed T2 cells at a ratio of 2:1 for 3.5 h at 37°C in the presence of an anti-CD107a antibody. More frequent $CD107a^+$ cells were observed when CTLs were co-cultured with T790M-A peptide-pulsed T2 cells compared to HIV-peptide-pulsed T2 cells, and $CD8^+$ $CD107a^+$ cells were sorted as a purified, peptide-specific CTL line using a FACSAria II cell sorter (Fig. 2C). A purified T790M-A-specific CTL line was established from healthy donor 3.

Cross-reactivity of the T790M-A-specific CTL line with other EGFR T790M-derived peptides. To assess its cross-reactivity with other EGFR T790M-derived peptides, the T790M-A-specific CTL line was cultured with T2 cells pulsed with each peptide, and IFN-γ production was measured by ELISPOT assay. The T790M-A-specific CTL line specifically recognized T2 cells pulsed with T790M-A (789-797) but not non-peptide-pulsed T2 cells. The T790M-A-specific CTL line did not recognize T2 cells pulsed with the T790M-A (789-797) wild-type (ITQLMPFGC) peptide. Also, T2 cells pulsed with T790M-B, -D, and -E were not recognized by the T790M-A-specific CTL line (Fig. 3A). However, the T790M-A-specific CTL line showed cross-reactivity with T2 cells pulsed with T790M-C.

Next, we evaluated the cytolytic activity of the T790M-A-specific CTL line against cognate peptide-pulsed T2 cells. The T790M-A-specific CTL line specifically lysed T790M-A peptide-pulsed T2 cells but not HIV-peptide-pulsed T2 cells (Fig. 3B). These results suggest that the T790M-A-specific CTL line showed cross-reactivity against some EGFR

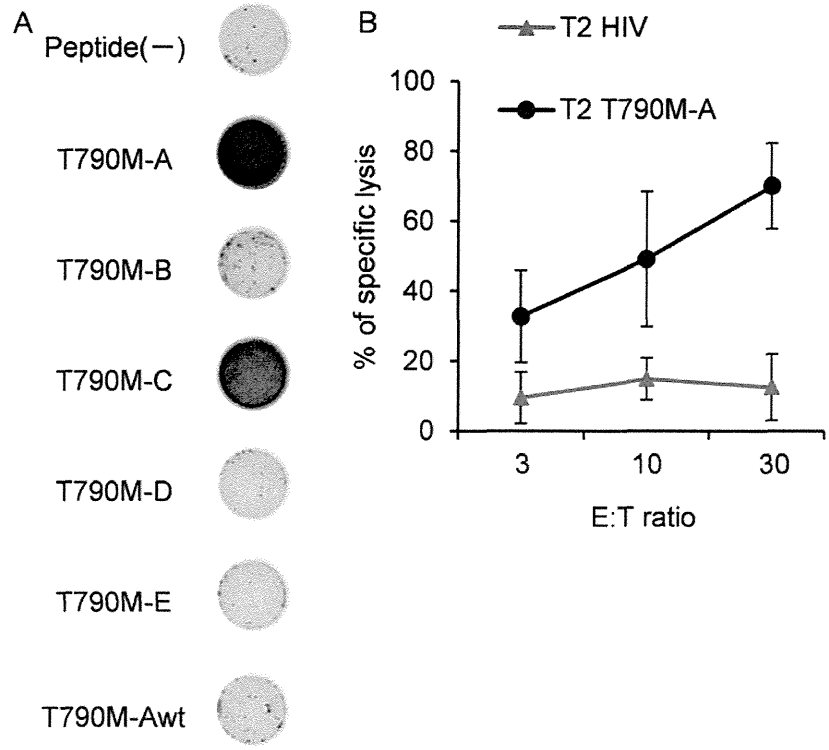

Figure 3. Cross-reactivity of the T790M-A-specific CTL line with threonine to methionine change at codon 790 of EGFR (EGFR T790M)-derived peptides. (A) Interferon-γ (IFN-γ) enzyme-linked immuno spot (ELISPOT) assay against T2 cells pulsed with each peptide. T2 cells pulsed with EGFR T790M-derived peptides (EGFR T790M-A, -B, -C, -D, -E, and Awt) were used as the target (effector $1x10^4$ cells/well, target $1x10^4$ cells/well). The assays were carried out in duplicate wells, and representative data are shown. (B) Cytotoxicity of the T790M-A-specific CTL line against T790M-A peptide-pulsed T2 cells. HIV-peptide-pulsed T2 cells were used as a negative control. Data are presented as means ± SD of three independent batches.

T790M-derived peptides, but not the corresponding wild-type EGFR-derived peptide. This cross-reactivity seems to be favorable for efficacy against EGFR T790M$^+$ cancer cells.

The T790M-A-specific CTL line recognizes and lyses HLA-A2$^+$ T790M$^+$ NCSLC cells. Next, we assessed the ability of the T790M-A-specific CTL line to recognize the HLA-A2$^+$ T790M$^+$ NCSLC cell line. This CTL line was incubated with 11-18 (T790M$^-$, HLA-A2$^+$), T790M-A-pulsed 11-18, H-1975 (T790M$^+$ HLA-A2$^-$), or H-1975-A2 (T790M$^+$ HLA-A2$^+$), and IFN-γ production was evaluated. We confirmed that the T790M-A-specific CTL line recognized peptide-pulsed 11-18 and H-1975-A2, but not 11-18 and H-1975, cells by IFN-γ ELISPOT assay (Fig. 4A). Similar data were obtained using CTLs from healthy donor 1 stimulated with T790M-A peptide-pulsed DC and aAPC-A2 *in vitro*, which were not purified by the CD107a assay (data not shown).

To evaluate the function of the T790M-A-specific CTL line against H1975-A2, a CD107a assay was performed. CD107a$^+$ cells were detected more frequently in culture with H-1975-A2 than with H-1975 cells (Fig. 4B).

Finally, we investigated the cytotoxic activity of the T790M-A-specific CTL line against H-1975-A2. Target cells were labeled with calcein-AM and co-cultured with the effector cells for 4-6 h. The T790M-A-specific CTL line showed cytotoxic activity against H1975-A2 cells, but not H1975 cells (Fig. 4C). These results suggest that the T790M-A-specific CTL line can recognize NSCLC cells harboring the EGFR T790M mutation in an HLA-A2-restricted manner.

Discussion

Mutated antigens associated with tumor cell progression and survival or drug resistance represent novel targets for cancer vaccine therapy. Warren *et al* evaluated computationally the antigenic potential of somatic mutations that occur in human cancers (26). They showed that several gene mutation-derived epitopes have immunogenic potential, at least computationally. Moreover, point mutations within the ABL kinase domain of the *BCR-ABL* gene are the most common causes of resistance to imatinib in chronic myeloid leukemia (CML) patients (27). Cai *et al* reported that the mutated *BCR-ABL* gene was associated with a TKI-resistance-generated CTL epitope in CML patients (28). These results suggest new immunotherapeutic approaches based on a TKI-resistant mutation-derived neoantigen. That is, mutations associated with acquired resistance to TKI therapy can be targeted by immune-based treatment strategies. This strategy may be an option to treat the gene mutation-mediated drug-resistant cancer cells. In the present study, we demonstrated the immunogenicity of antigens from mutated EGFR that are involved in TKI resistance in NCSLC.

TAAs can be classified into several categories, such as cancer-testis (CT) antigens, overexpressed antigens, differentiation antigens, and mutated antigens. Of these, only mutated antigens are unique, because they are not expressed in normal tissues. Previous reports have shown that peptide vaccine therapy can occasionally induce ineffective CTL responses, contrary to expectations (29-31). One possibility is

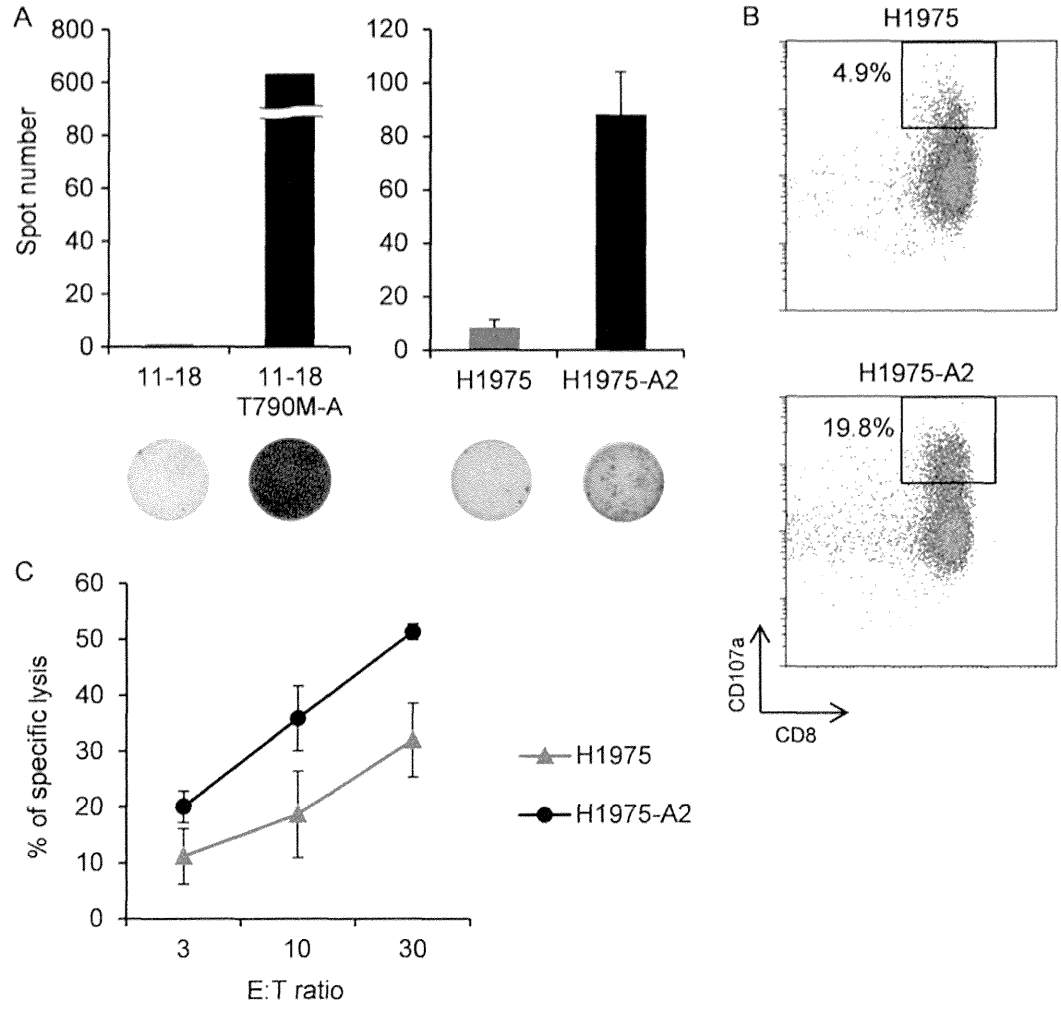

Figure 4. Reactivity of the T790M-A-specific CTL line against non-small-cell lung cancer (NSCLC) cells with or without the T790M mutation. (A) Interferon-γ (IFN-γ) enzyme-linked immuno spot (ELISPOT) assay results for the T790M$^+$ and T790M$^-$ NSCLC lines. Left: 11-18 and T790M-A peptide-pulsed (10 μg/ml) 11-18 cells were used as the targets (effector $1x10^5$ cells/well, target $1x10^5$ cells/well). Right: H-1975 and H1975-A2 cells were used as the targets (effector, $5x10^4$ cells/well; target, $5x10^4$ cells/well). The bars indicate the IFN-γ ELISPOT counts. (B) CD107a assay of the T790M$^+$ NSCLC line (E:T = 2:1). CD8$^+$ CD107a$^+$ cells were gated. (C) Cytotoxicity against the T790M$^+$ NSCLC cell line at the indicated effector/target ratios. Data are presented as means ± SD of three independent batches.

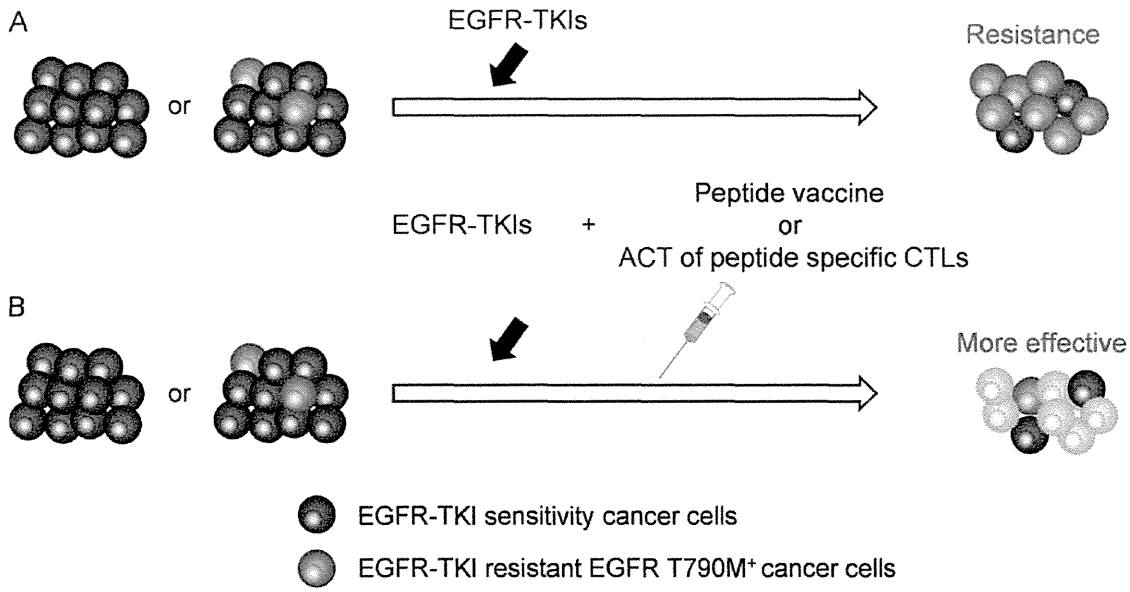

Figure 5. Combination therapy of epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) with T790M-targeted immunotherapy. (A) Generally, cancer cells develop acquired resistance to EGFR-TKI. (B) TKI-resistant cells harboring the T790M mutation were targeted by immunotherapy. This combination therapy may be effective against cancers with and without the threonine to methionine change at codon 790 of EGFR (EGFR T790M) mutation.

that the induced antigen-specific CTLs have a low affinity, and thus recognize only target cells pulsed with high concentrations of the peptide and not naturally presented epitopes on tumor cells. Several EGFR-derived CTL epitopes have been identified (32,33); however, the frequency of high-avidity EGFR-specific CTLs seems to be low in patients with EGFR-expressing cancers, because EGFR is a self-antigen that induces tolerance. The ability of low-avidity CTLs to recognize antigen-expressing tumor cells is considered to be weak. However, mutation-derived antigens are not self-antigens; thus, they would not be expected to induce immunotolerance, and so may have high immunogenicity. Indeed, in melanoma patients who experienced dramatic therapeutic effects after adoptive cell therapy with tumor-infiltrating lymphocytes (TILs), the mutated antigen-derived epitope was immunodominant and was recognized by tumor-reactive T cells (34,35).

In the present study, BIMAS was used to select EGFR T790M-derived candidate peptides that bind to HLA-A*02:01 according to computer algorithms, and T790M-A-specific CTLs could be induced from PBMCs of all four healthy donors by stimulation with peptide-pulsed DCs and aAPCs. Amino acid substitution of anchor residues (at position 2 and the C-terminus for HLA-A2) can alter the binding affinity (36-38). Leucine and methionine are the preferred anchor residues at position 2 of HLA-A2 (36,37). T790M-A (IMQLMPFGC) harbors a substitution of threonine to methionine at the anchor site, which confers immunogenicity. Also, valine and leucine are the preferred anchor residues at the C-terminus (36,37).

Then, we designed the modified peptides, T790M-D (IMQLMPFGV, substitution of cysteine to valine at the C-terminus) and T790M-E (IMQLMPFGL, substitution of cysteine to leucine at the C-terminus). These peptides bound to the HLA-A*02:01 molecule strongly (Fig. 1), but could not induce specific CTLs. T790M-D and -E are not self-antigens, being similar in this respect to T790M-A; this difference may be due in part to the difference in the frequency of peptide-specific CTL precursors. To confirm that the predicted candidate peptides are naturally presented peptides on tumor cells, peptide-specific CTL clones or lines induced by the peptides must recognize the tumor cells. A mass spectrometry (MS)-based method facilitates identification of peptide presentation by tumor cells (39). In this study, we confirmed the peptide-specific recognition of tumor cells by a peptide-specific CTL line, but not a CTL clone. However, CTL lines may contain distinct CTL clones that recognize irrelevant peptides, leading to apparent tumor reactivity (40). To avoid misleading tumor recognition and to evaluate the antigen-specific response of a CTL line, we used a peptide-specific CTL line established by CD107a sorting. An IFN-γ ELISPOT assay suggested that the specific CTL line recognized NSCLC cells harboring the EGFR T790M mutation in an HLA-A*02:01-restricted manner.

The T790M-A-specific CTL line did not show activity against the corresponding wild-type peptide. This suggests that EGFR T790M-targeted immunotherapy has no effect on NSCLC prior to EGFR-TKI treatment, with the exception of any pre-existing population of T790M-harboring cells, at least theoretically. Thus, consideration of combination therapy, EGFR-TKI and EGFR T790M-targeted immunotherapy, seems reasonable. Several studies have suggested that combination therapy could improve the efficacy of cancer immunotherapy. For instance, some chemotherapeutic agents can lead to upregulation of TAA expression or improvement of tumor cell resistance to specific CTLs (41). Use of an EGFR-TKI or anti-EGFR antibody augments the IFN-γ-induced expression of MHC classes I and II by A431 malignant human keratinocytes (42). Moreover, gefitinib improved the cytotoxic activity of natural killer cells against H1975 by modulating the interaction between NK cells and cancer cells, and by inhibiting STAT3 expression (43). These results indicate that the combination of EGFR-TKI and immunotherapy may have synergistic activity against NSCLC cells. The concept of combination therapy is shown in Fig. 5. Adding EGFR T790M-targeted immunotherapy to EGFR-TKI treatment could control the progression of cancer cells harboring T790M.

Yamada *et al* reported two HLA-A2-restricted EGFR T790M-derived CTL epitopes (790-799 MQLMPFGCLL and 788-798 LIMQLMPFGCL) (44). In addition to these epitopes, we identified the HLA-A*02:01-restricted CTL epitope T790M-A (789-797 IMQLMPFGC). We found that a T790M-A-specific CTL line established from human PBMCs had the ability to recognize and lyse the HLA-A*02:01$^+$ T790M$^+$ NCSLC cell line, and importantly, did not show cross-reactivity with the corresponding wild-type EGFR peptide. These results suggest that the EGFR T790M-A-specific CTL line recognizes single amino acid substitutions, leading to a low level of auto-immune reaction. The combination of an EGFR-TKI and T790M-targeted immunotherapy may be useful for treatment of NSCLC with the T790M mutation.

Acknowledgements

We thank Professor Seiji Yano for providing the NSCLC cell line H1975, Dr Tetsuro Sasada for providing the NSCLC cell line H1975-A2, and Professor Naoto Hirano for providing aAPC-A2. This study was supported in part by the National Cancer Center Research and Development Fund (25-A-7), as well as Research for the Promotion of Cancer Control Programmes, Research on Applying Health Technology, and Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour, and Welfare, Tokyo, Japan.

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Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes

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Received September 7, 2014; Accepted October 17, 2014

DOI: 10.3892/ijo.2014.2737

Abstract. Novel treatment modalities are required urgently in patients with hepatocellular carcinoma (HCC). A vaccine that induces cytotoxic T lymphocytes (CTLs) is an ideal strategy for cancer, and glypican-3 (GPC3) is a potential option for HCC. Blocking the programmed death-1 (PD-1)/PD-L1 pathway is a rational strategy to overcome tumor escape and tolerance toward CTLs. In the present study, we investigated whether anti-PD-1 blocking antibodies (αPD-1 Ab) enhanced the number of vaccine-induced peptide-specific CTLs in peripheral blood mononuclear cells (PBMCs) following the administration of GPC3 peptide vaccine to both patients and in a mouse model. The inhibitory receptor PD-1 was highly expressed in *ex vivo* GPC3-specific CTLs isolated from the PBMCs of vaccinated HCC patients. *In vitro*, interferon-γ induced PD-L1 expression in liver cancer cell lines. In addition, PD-1 blockade increased the number of GPC3-specific CTLs, which degranulate against liver cancer cell lines. *In vivo* experiments using tumor-bearing mouse models showed that the combination therapy of peptide vaccine and αPD-1 Ab suppressed tumor growth synergistically. PD-1 blockade increased the number of peptide-specific tumor-infiltrating T cells (TILs) and decreased the expression of inhibitory receptors on TILs. This study demonstrated that PD-1/PD-L1 blockade augmented the antitumor effects of a peptide vaccine by increasing the immune response of vaccine-induced CTLs, and provided a foundation for the clinical development of a combination therapy using a GPC3 peptide vaccine and αPD-1 Ab.

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Abbreviations: HCC, hepatocellular carcinoma; CTL, cytotoxic T lymphocyte; GPC3, glypican-3; PD-1, programmed death-1; PBMC, peripheral blood mononuclear cell; HLA, human leukocyte antigen; IFN-γ, interferon-γ; MHC, major histocompatibility complex

Key words: programmed death-1, cytotoxic T lymphocyte, peptide vaccine, glypican-3, hepatocellular carcinoma

Introduction

Antigen-specific cancer immunotherapy using the induction of tumor-specific reactions without autoimmunity is a potentially attractive option for the treatment of cancer. However, immunotherapy for hepatocellular carcinoma (HCC) is still in the preclinical or early clinical trial phases (I and II) of development (1,2). Glypican-3 (GPC3), a carcinoembryonic antigen, is overexpressed in 72-81% of HCC cases, and is correlated with poor prognosis; therefore, it is an ideal target for HCC (3-7). Recently, a phase I clinical study of a GPC3-derived peptide vaccine reported its safety and efficacy for the treatment of advanced HCC (8). Although vaccine-induced GPC3-peptide-specific cytotoxic T lymphocytes (CTLs) are often tumor reactive *in vitro* (9) and correlate with overall survival, no complete response was observed when GPC3 peptide vaccination was used as monotherapy in patients with advanced HCC (8).

Programmed death-1 (PD-1) is expressed on activated T and B cells, and elicits inhibitory signals (10). Its ligand PD-L1 is member of the B7 family, and interacts with PD-1 (11). Several studies have shown that the PD-1/PD-L1 pathway plays a critical role in compromised tumor immunity (12,13). PD-1 antibody blockade exerts antitumor effects in clinical trials (14,15). High expression levels of PD-1 on T cells, both in tumor-infiltrating lymphocytes (TILs) and peripheral blood mononuclear cells (PBMCs), were correlated with poor prognosis in HCC patients after surgical resection (16). In addition, PD-L1 expression in HCC was correlated with tumor aggressiveness and postoperative recurrence (17).

In animal models, PD-1 blockade exerts synergistic effects with various tumor vaccines to enhance tumor antigen-specific T cell responses and suppress tumors *in vivo* (18-20). It was reported that melanoma vaccine-induced CTLs become exhausted, which could be reversed by blocking the inhibitory pathways (21). However, a study evaluating the combination

of a cancer vaccine and an anti-PD-1 blocking antibody (αPD-1 Ab) for HCC has not been conducted. Therefore, the aim of this study was to investigate whether αPD-1 Ab would enhance the antitumor effects of a peptide vaccine by analyzing CTLs isolated from the PBMCs of vaccinated patients, as well as from a mouse model.

Materials and methods

Patient samples. Three clinical trials were conducted using GPC3-derived peptide vaccines. A phase I trial (n=33) was performed in patients with advanced or metastatic HCC (8) (University Hospital Medical Information Network Clinical Trials Registry; UMIN-CTR no. 000001395). Subsequently, a phase II trial was performed using a GPC3-derived peptide vaccine as an adjuvant therapy in patients with HCC (UMIN-CTR: 000002614, on-going). Finally, a pilot study of liver biopsies taken before and after GPC3 peptide vaccination is being performed for advanced HCC (UMIN-CTR: 000005093, on-going). These trials were approved by the Ethics Committee of the National Cancer Center, Japan, and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All patients were enrolled after providing written informed consent. Patients were injected intradermally with HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) or HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide vaccines emulsified with incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC).

Peripheral blood (30 ml) was obtained at the National Cancer Center Hospital East. PBMCs were isolated using standard Ficoll density gradient centrifugation from buffy coats. The remaining PBMCs were used after immunological monitoring in clinical trials. The immunological analyses were approved by the Ethics Committee of the National Cancer Center, Japan.

Cell lines. The human liver cancer cell lines SK-Hep-1 ($GPC3^-$, HLA-A*02:01/A*24:02), SK-Hep-1/GPC3 ($GPC3^+$, HLA-A*02:01/A*24:02), and HepG2 ($GPC3^+$, HLA-A*02:01/A*24:02) were available in our laboratory and were used as the target cells (6,9). SK-Hep-1/GPC3 is an established stable GPC3-expressing cell line that was transfected with the human GPC3 gene, whereas SK-Hep-1/vec is an established counterpart cell line that was transfected with an empty vector. The mouse lymphoma cell line RMA (OVA-, $H\text{-}2K^b$) was provided by Dr Yasuharu Nishimura (Kumamoto University, Japan). Cells were cultured at 37°C in RPMI-1640 or DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2.

Synthetic peptides and cytokines. The peptides used in this study were as follows: HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide (American Peptide Co.), HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) peptide (American Peptide Co.), HLA-A*02:01-restricted human immunodeficiency virus $(HIV)_{77-85}$ (SLYNTYATL) peptide (ProImmune), and $H\text{-}2K^b$-restricted ovalbumin $(OVA)_{257-264}$ (SIINFEKL) peptide (AnaSpec). The peptides were dissolved and diluted in 7% $NaHCO_3$ or dimethyl sulfoxide (DMSO). Where appropriate, liver cancer cell cultures were treated with 100 U/ml recombinant interferon (IFN)-γ (PeproTech).

Ex vivo Dextramer staining and flow cytometry. PBMCs were stained using HLA-A*02:01 Dextramer-RPE [$GPC3_{144-152}$ (FVGEFFTDV), HIV_{19-27} (TLNAWVKVV) or negative control; Immudex] and HLA-A*24:02 Dextramer-RPE [$GPC3_{298-306}$ (EYILSLEEL), $HIV_{583-591}$ (RYLKDQQLL); Immudex] for 15 min at room temperature, followed by anti-CD8-FITC (clone T8, Beckman Coulter), anti-PD-1-APC (clone EH12.2H7, BioLegend), or isotype control-APC (clone MOPC-21, BioLegend) for 20 min at 4°C. Flow cytometry was performed using a FACSCanto II (BD Biosciences).

Blocking antibody. GPC3 peptide-specific CTL clones were established from PBMCs as described previously (9). The CTL clones were cultured in AIM-V medium (Life Technologies) supplemented with 10% human AB serum in the presence of 10 μg/ml anti-PD-1 (clone J116, eBioscience) or 10 μg/ml control (clone MOPC-21, BioXcell) monoclonal antibodies for 2 days.

CD107a assay. GPC3 peptide-specific CTL clones were incubated with SK Hep-1/vec pulsed with $GPC3_{144-152}$ or HIV_{19-27} peptide and HepG2 at a 1:1 ratio for 3.5 h at 37°C. CTL clones were stained with anti-CD107a-APC (clone LAMP-1, BD Bioscience) during the incubation period, followed by anti-CD8-FITC (clone LT8, ProImmune) for 20 min at 4°C.

Mice. Female C57BL/6 mice (6-8 weeks old) were purchased from Japan Charles River Laboratories (Yokohama, Japan), and were maintained under specific pathogen-free conditions. The Animal Research Committee of the National Cancer Center, Japan, approved all studies. All animal procedures were performed according to the guidelines for the Animal Research Committee of the National Cancer Center, Japan. Ether was used for mouse euthanasia and anesthesia.

In vivo tumor growth inhibition assays. It was reported previously that intratumoral (i.t.) injection of $OVA_{257-264}$ peptide (SIINFEKL) effectively inhibited OVA-negative tumor growth and survival in a peptide vaccine model using C57BL/6 mice (22). RMA cells ($1x10^5$ cells/100 μl PBS) were implanted on the backs of C57BL/6 mouse on day 0. They were then injected with 50-μg peptide mixed with an equal volume of incomplete Freund's adjuvant (IFA, Montanide ISA-51VG; SEPPIC) on days 7 and 14. The total volume of injected vaccine solution was 100 μl in all experiments. For *in vivo* therapeutic experiments, anti-mouse PD-1 (clone 4H2) and control Ab (clone MOPC-21, BioXcell) were provided by Ono Pharmaceutical Co., Ltd. The anti-mouse PD-1 Ab (clone 4H2) used in the present study is a chimeric rat Ab containing the murine IgG1 Fc region (23). Anti-PD-1 or control Abs (200 μg/day) were injected intraperitoneally (i.p.) on days 7 and 14. Tumor volume was monitored twice per week, and was calculated using the following formula: tumor volume (mm^3) = a x b x b x 0.5, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. Mouse health, behavior and mortality were checked daily. All mice were maintained until they showed signs of morbidity or the

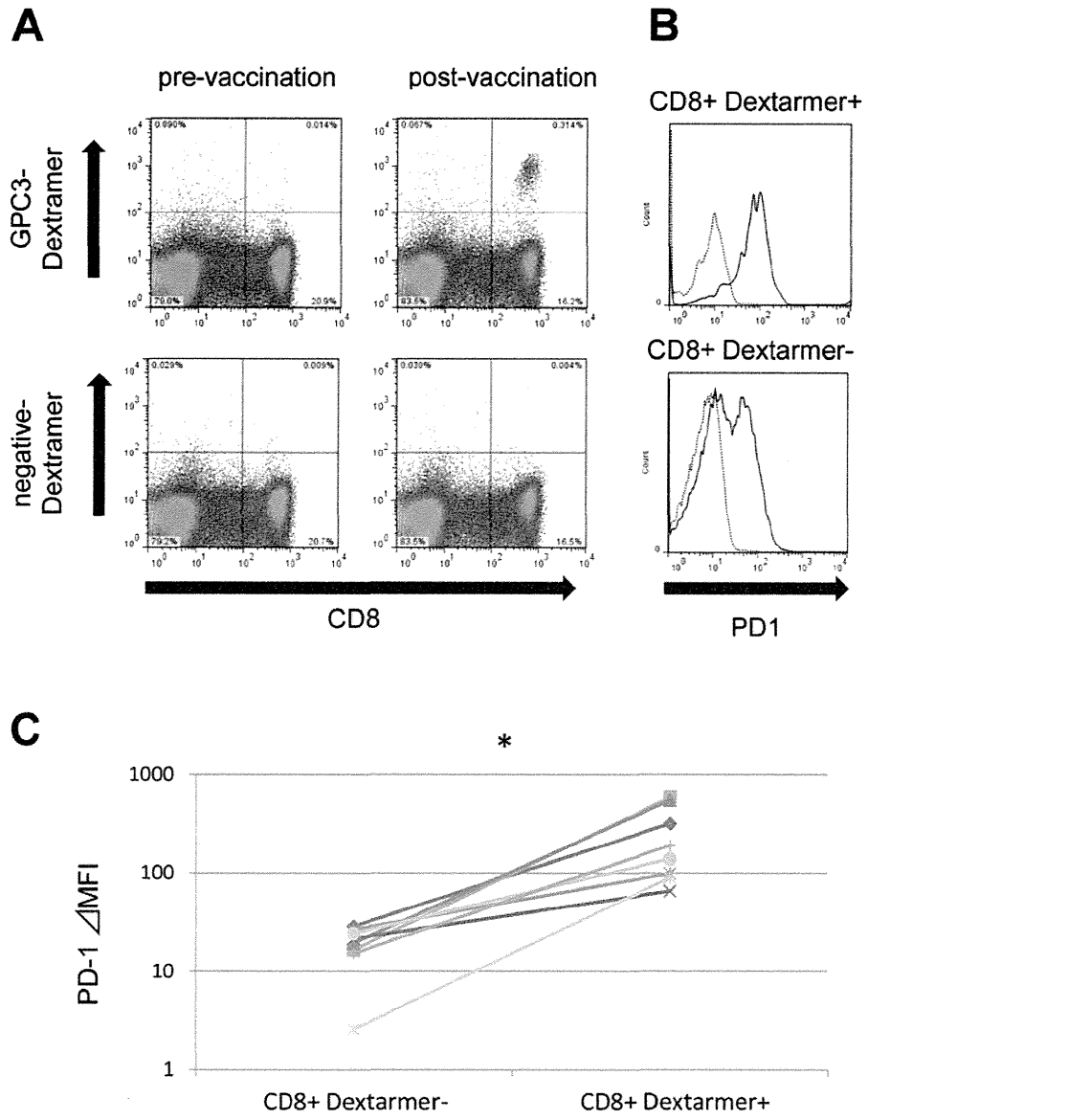

Figure 1. PD-1 expression on GPC3-specific CTLs after vaccination. (A) *Ex vivo* GPC3 Dextramer staining before and after vaccination in a representative case. The frequency of GPC3 peptide-specific CTLs is indicated as the percentage of the Dextramer-positive CTLs among PBMCs. (B) GPC3-specific CTLs were acquired by gating the CD8-positive/GPC3 Dextramer-positive population. The CD8-positive/GPC3 Dextramer-negative population was used as the control. (C) PD-1 expression on GPC3-specific CD8-positive/Dextramer-positive or -negative populations from eight patient specimens. ⊿, MFI, MFI using anti-PD-1 subtracted by that using isotype control. *P<0.05, n=8 using Wilcoxon's signed-rank test.

length or width of the tumors exceeded 30 mm, at which point they were sacrificed for reasons of animal welfare (22).

IFN-γ enzyme-linked immunospot (ELISPOT) analysis. The BD™ ELISPOT set (BD Biosciences) was used to assess the levels of IFN-γ, as described previously (24). Briefly, CD8-positive splenocytes ($5x10^5$) were added to the plate as effector cells. Then, either bone marrow-derived dendritic cells (BM-DCs; $1x10^5$) pulsed with OVA peptide (10 μg/ml; as target cells) or non-pulsed BM-DCs ($1x10^5$; as control cells) were added. The plate was then incubated for 37˚C, for 20 h in the presence of 5% CO_2. Spots were counted automatically using the Eliphoto system (Minerva Tech).

Isolation of mouse tumors and flow cytometry. The mice were sacrificed and the dorsal tumors were dissected, cut into small pieces, and digested with collagenase (1 mg/ml) for 20 min at 37˚C. After the intratumoral injection of $OVA_{257-264}$ peptide, tumor cells were isolated and stained with anti-mouse H-$2K^b$ bound to $OVA_{257-264}$ peptide (SIINFEKL)-PE (clone 25-D1.16, BioLegend) or isotype control-PE (MOPC-21, BioLegend). To analyze the local accumulation of antigen-specific CTLs in mice, isolated tumor cells including tumor-infiltrating lymphocytes were stained with H-$2K^b$ OVA Tetramer-PE [$OVA_{257-264}$ (SIINFEKL); MBL] for 30 min at room temperature. They were then incubated with anti-mouse CD8-FITC (clone KT15, MBL), anti-mouse PD-1-PE-Cy7 (clone 29F.1A12, BioLegend), anti-mouse CTLA-4-APC (clone UC10-4B, BioLegend), or anti-mouse LAG-3-PerCP-Cy5.5 (clone RTK2071, BioLegend) for 20 min at 4˚C.

Quantitative real-time PCR. The tumors implanted into mice were dissected. Total RNA was isolated from homogenized tumors using RNeasy mini kit (Qiagen) according to

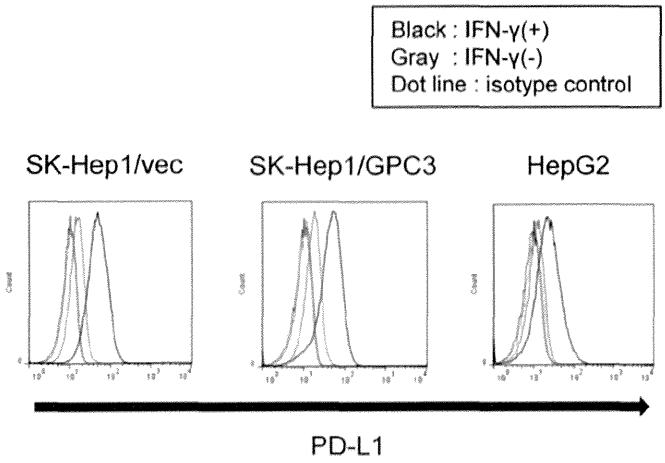

Figure 2. PD-L1 expression in liver cancer cell lines. Liver cancer cell lines were cultured with 100 U/ml IFN-γ for 24 h. PD-L1 expression was then analyzed using flow cytometry. Two independent experiments were performed, which yielded similar results.

the manufacturer's instructions. First-strand complementary deoxyribonucleic acid (cDNA) was synthesized using a PrimeScript® II first-strand cDNA Synthesis kit (Takara). Quantitative real-time PCR was then performed on an Applied Biosystems 7500 FAST Real-time PCR system using Power SYBR® Green (Applied Biosystems). We assessed the expression of the chemokines CXCL10, CXCL12, and CCL3, and compared them to *β-actin*. Data ware analyzed using delta-delta CT methods. Primer sequences of the chemokines were as described (25), and were purchased from Sigma Genosys.

Statistical analysis. All statistical analyses were performed using PASW Statistics software, version 18.0 (SPSS Inc.). Statistical significance was defined as a value of $P<0.05$ based on a two-tailed test.

Results

PD-1 expression ex vivo in GPC3 peptide-specific CTLs after vaccination in patients. To investigate whether vaccine-induced CTLs were affected by the PD-1/PD-L1 pathway, we measured the *ex vivo* expression of PD-1 on vaccine-induced GPC3-specific CTLs using flow cytometry with the GPC3-Dextramer. We used PBMCs obtained from eight patients during clinical trials of the GPC3 peptide vaccine. After vaccination, the frequency of GPC3-specific CTLs increased and could be detected *ex vivo*, as shown in the representative case 1 (Fig. 1A). GPC3-Dextramer-positive CD8 lymphocytes had a higher expression of PD-1 compared with GPC3-Dextramer-negative CD8 lymphocytes (Fig. 1B; representative case 1). In all eight patients with detectable GPC3-specific CTLs *ex vivo* after vaccination, PD-1 expression levels were significantly higher in GPC3-Dextramer-positive CD8 lymphocytes compared with GPC3-Dextramer-negative CD8 lymphocytes (Fig. 1C). Before vaccination, no GPC3-Dextramer-positive CD8 lymphocytes were detected *ex vivo*; therefore, PD-1 expression was not analyzed.

PD-1 blockade augments the GPC3-specific CTL clones that degranulate against liver cancer cell lines. SK-Hep1/vec, SK-Hep1/GPC3, and HepG2 liver cancer cell lines cultured with IFN-γ exhibited marked induction of PD-L1 on their surface (Fig. 2). This suggests that liver cancer cells are invaded by IFN-γ-producing CTLs via the PD-L1-mediated ligation of PD-1. Previously, several GPC3 peptide-specific CTL clones were established from PBMCs isolated from vaccinated patients. These clones exhibited cytotoxic activity

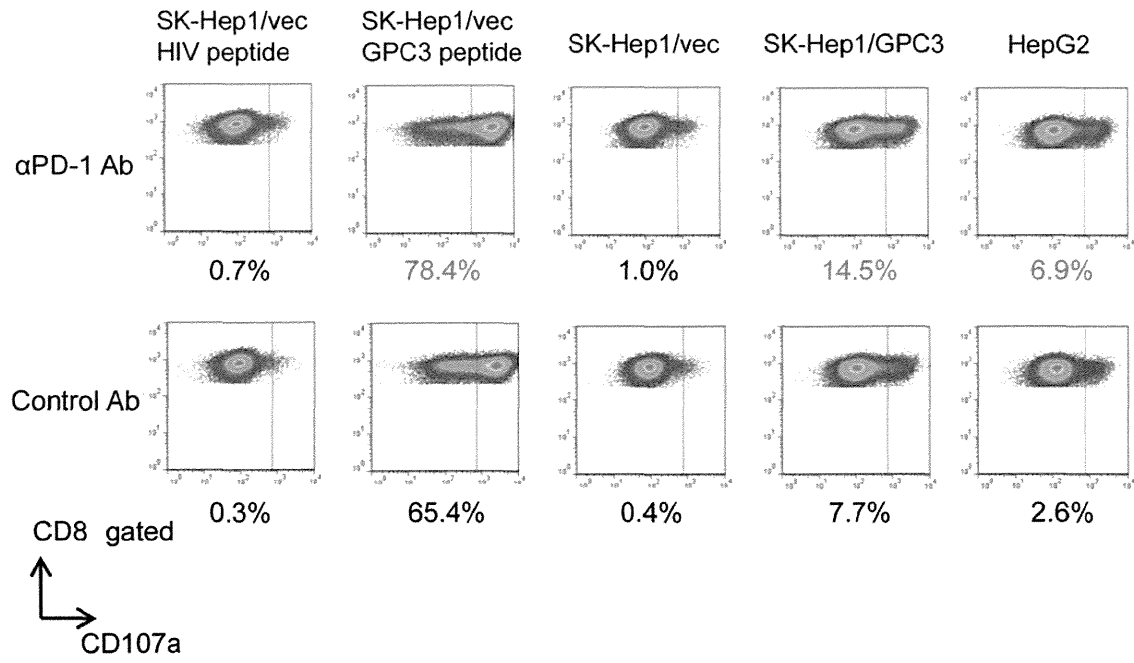

Figure 3. Blocking PD-1 increases GPC3-specific CTL clones that degranulate against liver cancer cell lines. The ratio of GPC3-specific CTL clones that externalized CD107a is shown below each column. The liver cancer cell lines used as the target cell are shown above each column. SK-Hep1/vec (GPC3$^-$) cells pulsed with peptide (1 μg/ml) were used as the target cells. The culture conditions are shown in rows. GPC3-specific CTL clones were acquired by gating the CD8-positive population. Two independent experiments were performed, which yielded similar results.

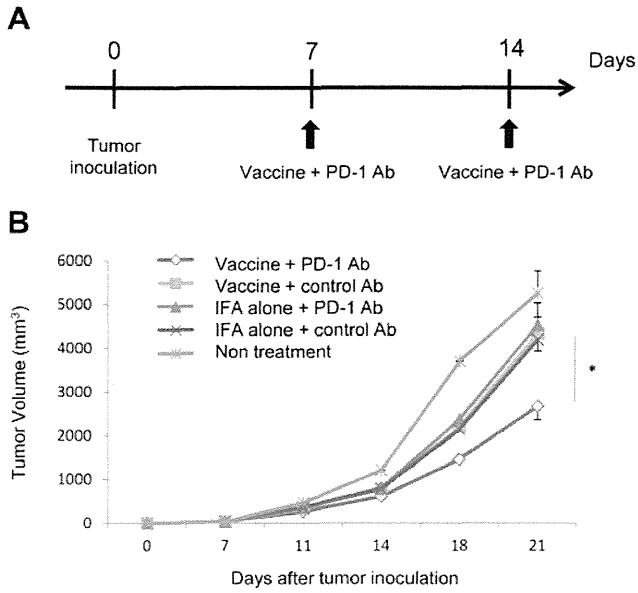

Figure 4. Peptide vaccine and αPD-1 Ab suppress tumor growth synergistically *in vivo*. (A) Mice implanted with RMA were treated with OVA peptide vaccine or IFA alone in combination with αPD-1 Ab or control Ab on days 7 and 14. (B) Tumor growth was expressed as mean tumor volume; bars, SE. Vaccine, OVA peptide emulsified with IFA; IFA alone, vehicle emulsified with IFA. *P<0.05, n=10 using Tukey's test. Two independent experiments were performed, which yielded similar results.

against cancer cells expressing GPC3 endogenously (9,26). Therefore, the CD107a (lysosomal-associated membrane protein-1)-mediated externalization of GPC3 peptide-specific CTL clones was examined upon exposure to liver cancer cell lines. The externalization of CD107a could be a surrogate marker to identify the antigen-specific CTLs that degranulate against tumor cells (27). CTL clones mobilized CD107a in response to SK-Hep1/vec pulsed with $GPC3_{144-152}$ peptide, SK-Hep-1/GPC3, and HepG2 ($GPC3^+$, HLA-$A^*02:01^+$), but not in response to pulsed SK-Hep1/vec with HIV_{19-27} (Fig. 3). Furthermore, PD-1 blockade enriched the population of GPC3-specific CTLs that degranulated against only GPC3-positive liver cancer cell lines (SK-Hep1/vec pulsed with $GPC3_{144-152}$ peptide, SK-Hep1/GPC3 and HepG2). These results suggest that blocking the interaction between PD-1 and PD-L1 enhanced the antitumor effect of CTLs in liver tumor cells that evade CTLs via PD-L1 expression.

Combination of a peptide vaccine and αPD-1 Ab suppresses tumor growth in vivo synergistically. Intratumoral injection with $OVA_{257-264}$ peptide (SIINFEKL) effectively inhibited the growth of OVA-negative tumors in a mouse model treated with a peptide vaccine (22). Therefore, we performed *in vivo* therapeutic experiments using intratumoral OVA peptide vaccine and αPD-1 Ab in tumor implanted mice. Mice were implanted with RMA tumor cells on day 0, and established tumors (3-6 mm in diameter) were treated with OVA peptide emulsified with IFA (vaccine) or vehicle emulsified with IFA (IFA alone) in combination with αPD-1 Ab or control Ab on day 7. An additional dose of vaccine and αPD-1 Ab was administered on day 14 after tumor inoculation (Fig. 4A). On day 21, one mouse in the untreated group was dead, and all other mice were alive. The tumor volume of mice treated using the combination therapy of vaccine and αPD-1 Ab was significantly less than those treated with the appropriate control (Fig. 4B, n=10). Treatment with vaccine/control Ab or IFA alone/αPD-1 Ab did not inhibit tumor growth compared with IFA alone/control Ab treatment. These data suggest that the combination of peptide vaccine and αPD-1 Ab had a synergistic antitumor effect.

Vaccine and αPD-1 Ab treatment increases the number of peptide-specific CTLs within mouse tumors. The loading of injected peptide onto major histocompatibility complex (MHC) class I molecules in tumor cells *in vivo* was reported previously using IFN-γ ELISPOT assays (22). In the present study, RMA (OVA-, H-$2K^b$) tumor cells were inoculated onto the backs of C57/BL6 mice. When the tumor diameter reached 3-6 mm, 50 μg H-$2K^b$-restricted $OVA_{257-264}$ peptide was injected into the tumor. After 96 h, the tumors were dissected, cut into small pieces, and digested using collagenase. To investigate whether the injected peptide was loaded onto the MHC class I molecules in the tumor cells in a solid mass, flow cytometry using anti-mouse H-$2K^b$ bound to $OVA_{257-264}$ peptide was performed. The loading of H-$2K^b$-restricted $OVA_{257-264}$ peptide onto MHC class I of tumor cells was detected (Fig. 5A).

To evaluate the immunological response to intratumoral OVA peptide vaccine and αPD-1 Ab, the spleens and tumors of mice treated with the same schedule were analyzed as described previously (Fig. 4A). Peptide-specific immune responses were detected in the spleens of mice treated with intratumoral OVA peptide injection using IFN-γ ELISPOT assays (Fig. 5B). Mice that received the combination of intratumoral OVA peptide injection and αPD-1 Ab exhibited an increased number of OVA peptide-specific CTLs compared with those treated with control Ab on day 14 (n=10).

To obtain direct evidence that the combination of peptide vaccine and αPD-1 Ab led to the local accumulation of antigen-specific CTLs, an OVA tetramer assay was performed in mice. OVA-tetramer-positive CD8 lymphocytes could be detected within a tumor using flow cytometry on day 21. Mice that received the combination of OVA peptide vaccine and αPD-1 Ab had a significantly increased number of OVA peptide-specific CTLs compared with those treated with control Ab (Fig. 5C and D; n=8).

Inhibitory receptors on tumor-infiltrating T lymphocytes and the expression of chemokines. The expression of inhibitory receptors on peptide-specific CTLs at the tumor site was assessed to investigate the mechanism of CTL accumulation in the tumors of mice treated with the combination therapy of peptide vaccine and αPD-1 Ab. RMA-bearing mice were treated with intratumoral OVA peptide injection combined with αPD-1 Ab or control Ab, as described previously (Fig. 4A). The expression of PD-1, CTLA-4, and LAG-3 in OVA tetramer-positive CD8 lymphocytes within the tumor on day 21 was analyzed using flow cytometry. The expression of the inhibitory receptors PD-1 and CTLA-4 was decreased in OVA-tetramer positive CD8 lymphocytes in the αPD-1 Ab group compared with the control Ab group (Fig. 6A). However, αPD-1 Ab treatment did not decrease LAG-3 expression in OVA tetramer-positive CD8 lymphocytes.

The expression of chemokines within the tumor on day 21 was examined using quantitative real-time PCR. The expres-

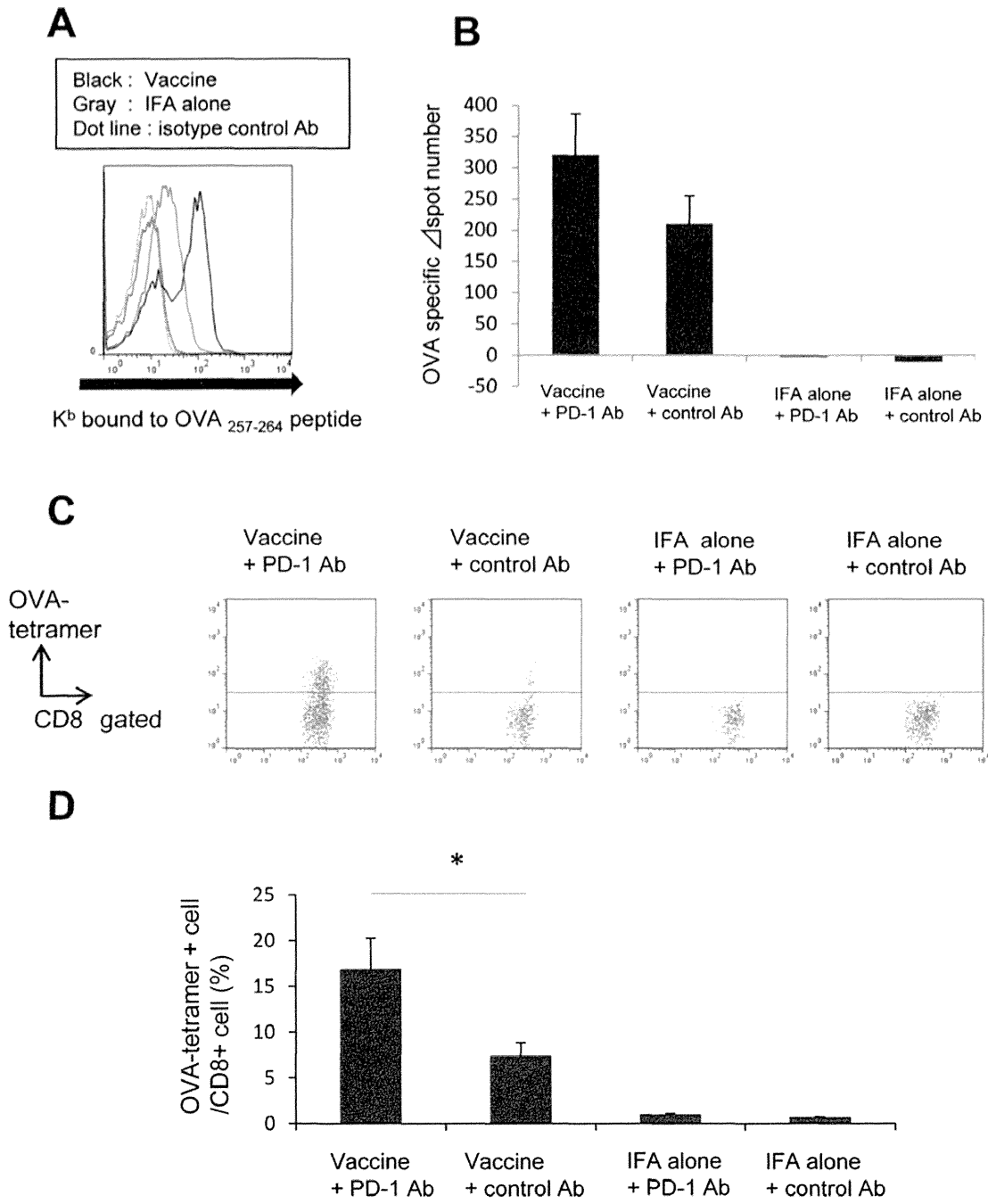

Figure 5. Blocking PD-1 enhanced the infiltration of vaccine-induced CTLs into the tumor. (A) Four days after the intratumoral injection with $OVA_{257-264}$ peptide, isolated RMA tumor cells were stained with anti-mouse H-$2K^b$ bound to $OVA_{257-264}$ peptide or isotype control and analyzed using flow cytometry. Data are presented from a single representative sample (n=3). (B) RMA-bearing mice were treated with OVA peptide vaccine or IFA alone in combination with αPD-1 Ab or control Ab. Spleen cells from treated mice were analyzed using an *ex vivo* IFN-γ ELISPOT assay on day 14. OVA-specific Δspot number, spot number of $OVA_{257-264}$ peptide pulsed BM-DC subtracted by non-pulsed BMDC. Data are presented as means ± SEM (n=10). (C) Tumor-infiltrating T lymphocytes were analyzed using flow cytometry on day 21. Representative plots of OVA tetramer-positive, CD8-positive TILs in the tumors treated with the combination therapy of intratumoral OVA peptide injection and αPD-1 Ab. (D) The percentages of OVA tetramer-positive cells in CD8-positive TILs are shown from three independent experiments using 2-3 mice per group. Data are presented as means ± SEM. $^*P<0.05$, n=8 using Student's t-test.

sion of the chemokine CCL3 was elevated in mice treated with the combination of intratumoral OVA peptide injection and αPD-1 Ab (Fig. 6B). The expression of the chemokines CXCL10 and CXCL12 was unchanged.

Discussion

Many tumor antigens have been identified in HCC, and their potential clinical utility for the development of cancer-specific immunotherapy has been investigated (28-31). GPC3 is a promising target of antigen-specific immunotherapy because it is overexpressed specifically in human HCC (3,4). In addition, it promotes tumor growth by stimulating canonical Wnt signaling (32) or the Hippo pathway (33). A phase I clinical trial of a GPC3-derived peptide vaccine in patients with advanced HCC showed that it had the potential to improve overall survival, which was associated with vaccine-induced CTLs (8). However, the antitumor effects of the peptide-based tumor

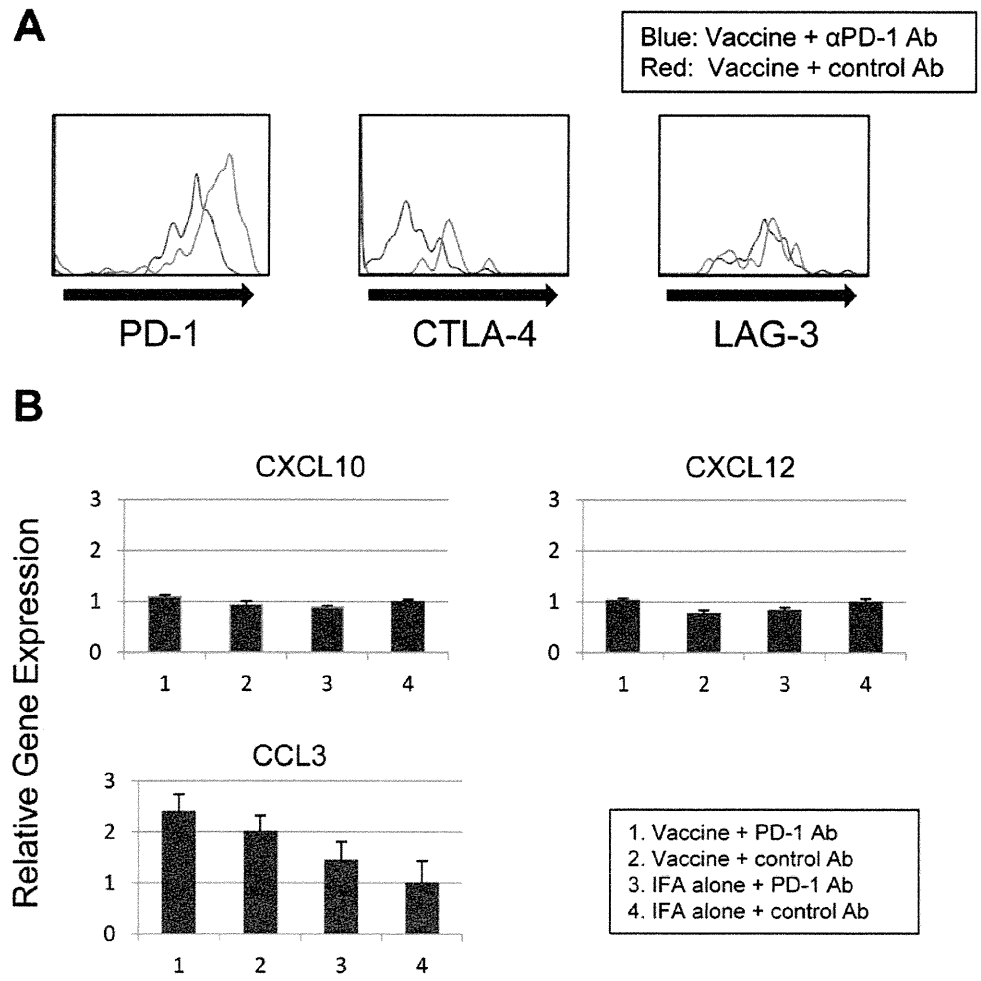

Figure 6. Changes in the expression of inhibitory receptors on tumor-infiltrating T lymphocytes and chemokines at the site of tumors treated using the combination therapy with peptide vaccine and αPD-1 Ab. RMA-bearing mice were treated with intratumoral OVA peptide injection combined with αPD-1 Ab or control Ab. On day 21, mice were sacrificed and the tumors were isolated. (A) Histogram showing the expression of the inhibitory receptors PD-1, CTLA-4, and LAG-3 in OVA tetramer-positive CD8 lymphocytes in tumors from mice treated with intratumoral OVA peptide injection and αPD-1 Ab, as well as from mice treated with intratumoral OVA peptide injection and control Ab. Data are from a single representative sample (n=4-6). (B) The expression levels of chemokines in the tumor were analyzed using quantitative real-time PCR (n=3). Relative expression levels in tumors treated with IFA alone and control Ab were calculated as the control. Data are presented as means ± SEM. Two independent experiments were performed, which yielded similar results.

vaccine alone were not satisfactory in patients with advanced HCC (8,29-31). Several studies identified molecules associated with the tumor escape mechanism, such as PD-1/PD-L1, Fas/FasL, and Decoy receptor 3, which might explain the poor immunogenicity and limitations of the antitumor effects of cancer vaccines alone in patients with advanced HCC (16,17,34,35). Therefore, the present study examined whether blocking PD-1/PD-L enhanced the antitumor effects of peptide vaccines in HCC.

The inhibitory receptor PD-1, was upregulated in GPC3-specific CTLs of HCC patients vaccinated using GPC3 peptide, consistent with previous reports of melanoma vaccine trials (21,27). CTLs for some tumor antigens might not be detected directly *ex vivo*. The *ex vivo* analysis of antigen-specific CTLs from uncultured PBMCs could provide strong and novel immunological evidence in HCC vaccine trials. Fourcade *et al* reported that the upregulation of PD-1 and Tim-3 on CTLs was correlated with the expansion of melanoma-peptide vaccine-induced NY-ESO-1-specific CTLs (21). Further studies are necessary to understand the potential clinical efficacy of vaccine-induced CTLs.

In this experimental model, IFN-γ induced PD-L1 expression in liver cancer cell lines. It was also demonstrated that blocking PD-1 increased the number of GPC3-specific CTL clones that degranulate against these liver cancer cell lines *in vitro*. These results suggest that blocking the interaction between PD-1 and PD-L1 enhanced the antitumor effects of CTL in liver cancer cells that evaded CTLs by expressing PD-L1. In contrast, Xu *et al* reported that αPD-L1 or αCTLA-4 Abs did not enhance cytokine secretion and the proliferation of peripheral GPC3-specific $CD8^+$ T-cell from HCC patients significantly (36). Differences in the effects of blocking PD-1 and PD-L1 might account for the differences between spontaneous GPC3-specific CTLs and vaccine-induced CTLs.

The combination of a peptide vaccine with αPD-1 Ab enhanced tumor suppression and antigen-specific T cell infiltration into the tumors of mouse models. The exact mechanisms by which CTLs accumulate into tumors by blocking PD-1 are unclear. A previous study in a mouse model of adoptive cell transfer demonstrated that blocking PD-1 increased the production of CXCL10 by bone marrow-derived myeloid cells,