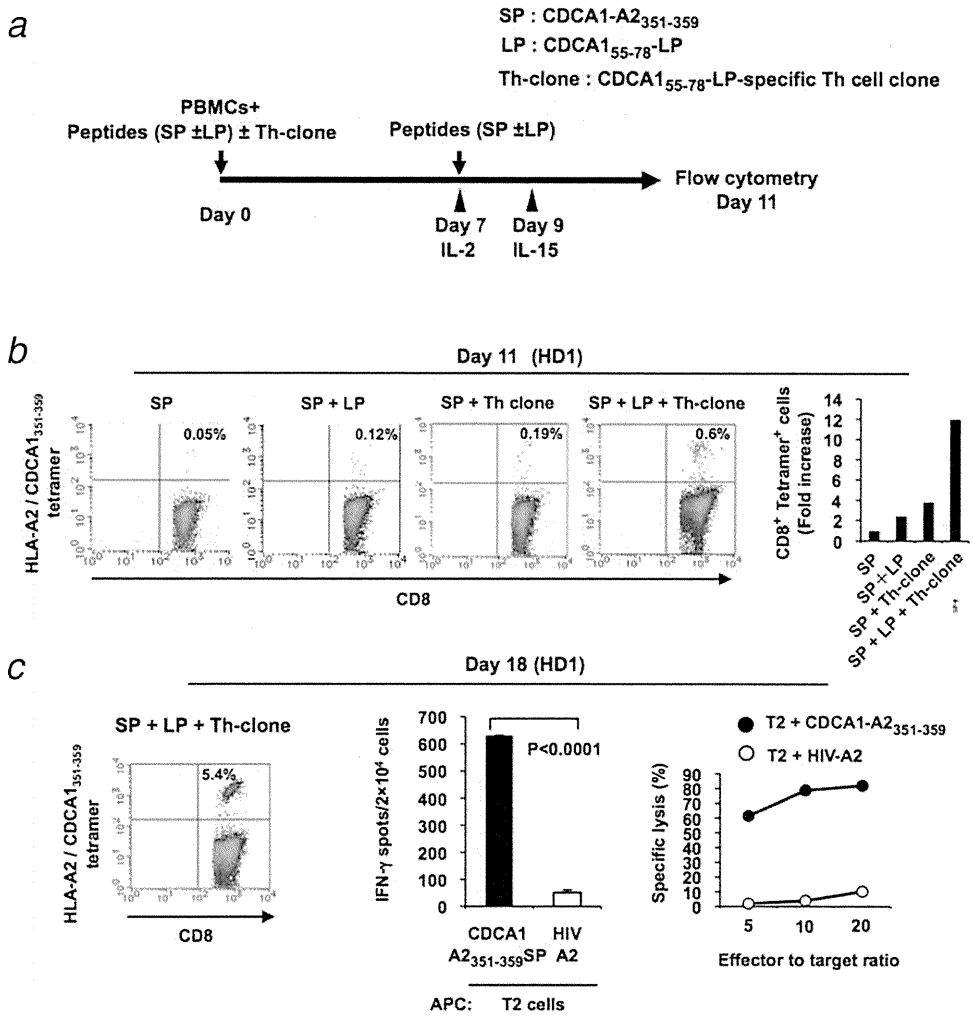

Figure 4. Enhanced induction of CDCA1-specific CTLs by stimulation using $CDCA1_{55-78}$-LP and $CDCA1_{55-78}$-LP-specific Th-clones. *a*: PBMCs from HLA-$A2^+$ and $DR4^+$ healthy donor-HD1, from which an HLA-DR4-restricted $CDCA1_{55-78}$-LP-specific $CD4^+$ Th-clone was generated, were cultured for 11 days with CDCA1-$A2_{351-359}$ (SP), SP + $CDCA1_{55-78}$-LP (LP), SP + $CDCA1_{55-78}$-LP-specific Th clone (Th-clone), or SP + LP + Th clone. On day 11, the cells were stained with HLA-A2/$CDCA1_{351-359}$ tetramer with an anti-human CD8 mAb and were analyzed by flow cytometry. *b*: Representative HLA-A2/$CDCA1_{351-359}$ tetramer staining (gated on $CD8^+$ T-cells) obtained from three independent experiments with similar results is shown. The increases (fold increase) in $CD8^+$ $tetramer^+$ cells are shown. *c*: The CDCA1-$A2_{351-359}$-specific CTL line (SP + LP + Th-clone) was re-stimulated with CDCA1-$A2_{351-359}$ SP and $CDCA1_{55-78}$-LP on day 14 (third stimulation). On day 18 of culture, cells were stained with tetramer (left panel). The number of IFN-γ-producing CTL was analyzed by ELISPOT assay (middle panel). ^{51}Cr-labeled and peptide-pulsed HLA-$A2^+$ T2-cells were used as target cells in a standard ^{51}Cr-release assay (right panel). The generated bulk CTLs were added at indicated effector/target ratio, and chromium release was measured 4 hr later.

alone or Control LP + SP. These results suggest that activated CDCA1-specific Th cells may be able to enhance induction of CDCA1-specific CTLs.

Presence of CDCA1-specifc Th cells in HNC patients vaccinated with CDCA1-$A24_{56-64}$ SP

In context of cancer immunotherapy, there is strong evidence suggesting that vaccines using restricted epitopes can result in broad $CD8^+$ T-cell responses to antigens not present in the vaccine.[35–37] Thus, we considered that CDCA1-specific Th cell responses might be efficiently induced by vaccination with a CDCA1-derived CTL-epitope peptide. To detect CDCA1-specific Th cell responses in patients, PBMCs isolated from 16 HNC patients vaccinated with CDCA1-$A24_{56-64}$ and 7 HNC patients before vaccination were collected. The donor characteristics are summarized in Table 1. After 1-week of *in vitro* stimulation of PBMCs with CDCA1-LPs, the frequency of individual CDCA1-LPs-specifc T-cells was detected by IFN-γ ELISPOT assay (Fig. 5*a*). PBMCs isolated from 10 healthy volunteers were used as controls. Responses were considered positive when the number of IFN-γ-secreting cells was at least twofold above the negative control.

Significant frequency of CDCA1-LP-specific immune responses were observed in HNC patients ($CDCA1_{39-64}$-LP,

Table 1. Clinical characteristics of HNC patients

Patient ID	Age/Sex	CDCA1-specific CD4$^+$ T-cell responses[1]				No. of vaccinations	Histologic subtype	*HLA-DRB1*	*HLA-DPB1*
		$CDCA1_{39-64}$-LP		$CDCA1_{55-78}$-LP					
CTR-8379 + CTR-8380									
Positive/Total, %		**14/19, 74%**		**13/19, 68%**					
CTR-8379		**Pre-vac.**	**Post-vac.**	**Pre-vac.**	**Post-vac.**				
Positive/Total, %		**2/6, 33%**	**7/10, 70%**	**2/6, 33**	**7/10, 70%**				
HNC10	61/M	n.t.	+	n.t.	−	60	Squamous cell carcinoma	*01:01/04:05*	*05:01/-*
HNC20	57/F	n.t.	−	n.t.	−	32	Squamous cell carcinoma	*01:01/09:01*	*02:01/05:01*
HNC26	70/M	n.t.	+	n.t.	+	24	Basaloid squamous cell carcinoma	*04:05/15:02*	*05:01/09:01*
HNC29	64/F	n.t.	+	n.t.	+	16	Squamous cell carcinoma	*09:01/14:54*	*03:01/05:01*
HNC31	69/F	n.t.	+	n.t.	+	16	Adenoid cystic carcinoma	*01:01/11:01*	*02:01/04:02*
HNC34	65/M	n.t.	+	n.t.	+	12	Squamous cell carcinoma	*08:03/15:02*	*02:01/05:01*
HNC35	85/F	n.t.	−	n.t.	+	8	Squamous cell carcinoma	*04:05/08:02*	*05:01/−*
HNC37	35/F	−	n.t.	−	n.t.	0	Adenoid cystic carcinoma	*04:05/15:02*	*02:01/05:01*
HNC38	56/M	−	n.t.	−	n.t.	0	Unknown	*09:01/−*	*02:01/05:01*
HNC39	77/M	−	+	−	+	8	Adenoid cystic carcinoma	*04:06/14:54*	*05:01/19:01*
HNC40	76/M	+	−	+	+	4	Squamous cell carcinoma	*01:01/09:01*	*04:02/05:01*
HNC41	51/F	+	n.t.	+	n.t.	0	Adenoid cystic carcinoma	*01:01/04:05*	*04:02/05:01*
HNC42	36/F	−	+	−	−	4	Unknown	*01:01/08:02*	*04:02/05:01*
CTR-8380		**Pre-vac.**	**Post-vac.**	**Pre-vac.**	**Post-vac.**				
Positive/Total, %		**0/1, 0%**	**5/6, 83%**	**0/1, 0%**	**5/6, 83%**				
HNC102	80/F	n.t.	-	n.t.	−	33	Squamous cell carcinoma	*15:02/−*	*02:01/09:01*
HNC103	78/F	n.t.	+	n.t.	+	20	Mutinous adenocarcinoma	*04:05/15:01*	*02:01/05:01*
HNC105	65/M	n.t.	+	n.t.	+	20	Angiosarcoma	*04:05/13:02*	*03:01/04:01*
HNC107	20/M	n.t.	+	n.t.	+	20	Osteosarcoma	*09:01/−*	*02:01/02:02*
HNC108	41/M	n.t.	+	n.t.	+	8	Osteosarcoma	*04:05/09:01*	*05:01/−*
HNC109	72/F	−	+	−	+	4	Verrucous carcinoma	*04:10/15:02*	*03:01/09:01*

CDCA1-specific T-cell responses measured by IFN-γ ELISPOT assay as detailed in the Materials and Methods. The experiments in 7 of 19 HNC patients (HNC10, 26, 34, 37, 38, 40, and 103) were performed in single wells. Number of vaccinations "0" indicates a patient before vaccination. The (+) and (−) indicate positive and negative responses. The underlined HLA-class II alleles encode HLA-class II-molecule presenting CDCA1-LP to Th cells in healthy donors (Fig. 1 and Supporting Information Fig. S3; *HLA-DRB1*04:05, DRB1*09:01, DRB1*15:02*, and *DPB1*02:01*). No., Number; CTR, Clinical Trials Registry; vac., vaccination; HNC, Head-and-neck cancer; M/F, male/female; LP, long peptide; n.t., not tested.

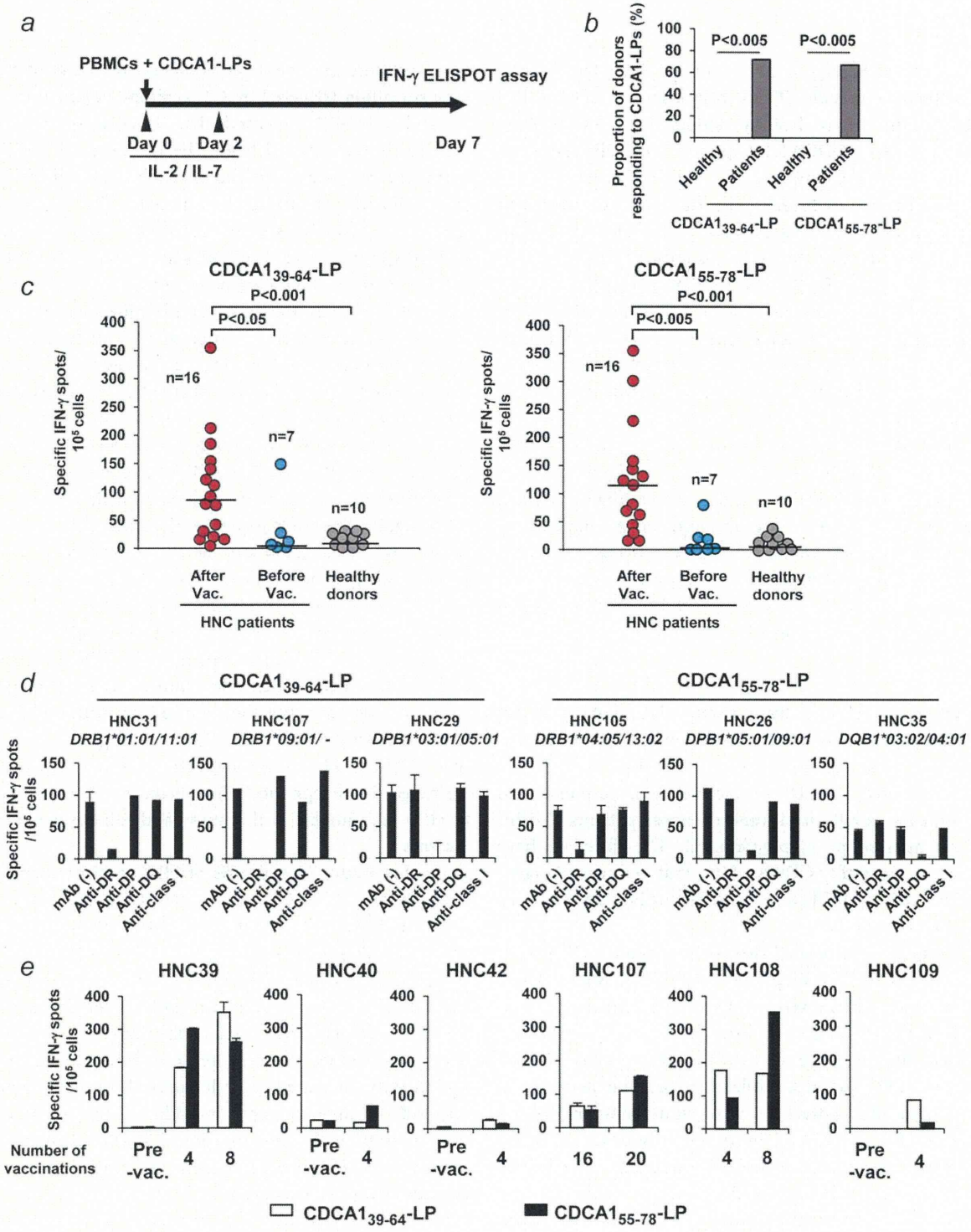

Figure 5. Presence of CDCA1-LPs-specific Th cells in PBMCs isolated from HNC patients vaccinated with CDCA1-A24$_{56-64}$ SP. *a*: After 1-week *in vitro* stimulation of PBMCs with a mixture of CDCA1$_{39-64}$-LP and CDCA1$_{55-78}$-LP, the frequency of individual CDCA1-LPs-specifc T-cells was detected by IFN-γ ELISPOT assay. *b*: HNC patients demonstrate elevated CDCA1-specific CD4$^+$ T-cell immunity compared to normal healthy individuals. Column graph showing proportion of healthy donors (control) and HNC patients responding to CDCA1-LPs. *p* values represent statistical results from Fisher's exact test. *c*: CDCA1-specific-Th cell responses were assessed in 16 HNC patients vaccinated with CDCA1-A24$_{56-64}$ SP (After Vac.), seven non-vaccinated patients (Before Vac.), and 10 healthy donors. The results represent specific IFN-γ spots after background subtraction. Each dot represents an individual donor. Horizontal lines denote median values, and *p* values represent statistical results from a nonparametric Mann-Whitney *U* test. The experiments in seven of 19 HNC patients (HNC10, 26, 34, 37, 38, 40 and 103) were performed in single well. *d*: HLA class II-restriction of the IFN-γ-producing T-cells. PBMCs stimulated with LPs for 1 week were restimulation with each CDCA1-LP in the presence of mAb specific to HLA-DR, -DP, -DQ, or HLA-class I. Six of 20 bar graph obtained from 12 HNC patients with similar results (HNC26, 29, 31, 34, 35, 39, 40, 42, 103, 105, 107 and 108) are shown. The experiments in five of 12 HNC patients (HNC26, 34, 40, 103 and 107) were performed in single well. CDCA1$_{39-64}$-LP; representative three bar graphs from 10 HNC patients (HNC26, 29, 31, 34, 39, 40, 42, 103, 107 and 108). CDCA1$_{55-78}$-LP; representative three bar graphs from 10 HNC patients (HNC26, 29, 31, 34, 35, 39, 40, 103, 105 and 108). *e*: The repeated CTL-epitope vaccinations induced (HNC39, 40, 42 and 109) or enhanced (HNC107 and 108) CDCA1-specific Th cell responses (CDCA1$_{39-64}$-LP, white bars; CDCA1$_{55-78}$-LP, black bars). The experiments in three of six HNC patients (HNC40, 108 and 109) were performed in single well. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

14 of 19, 74%; $CDCA1_{55-78}$-LP, 13 of 19, 68%; Fig. 5*b* and Table 1), but no specific IFN-γ responses to CDCA1-LPs were detected in the 10 healthy donors. In a few patients before vaccination, CDCA1-LP-specific Th cell responses were detectable ($CDCA1_{39-64}$-LP, two of seven, 29%; $CDCA1_{55-78}$-LP, two of seven, 29%; Table 1). Conversely, in many HNC patients after vaccination with CDCA1-$A24_{56-64}$ SP, CDCA1-LP-specific Th cell responses were detected ($CDCA1_{39-64}$-LP, 12 of 16, 75%; $CDCA1_{55-78}$-LP, 12 of 16, 75%; Table 1). The number of CDCA1-LP-specific IFN-γ-producing cells in patients after vaccination was significantly larger than in patients before vaccination and healthy donors (Fig. 5*c*). IFN-γ production by T-cells was significantly inhibited by addition of anti-HLA-class II mAb, but not by anti-HLA-class I mAb (Fig. 5*d*). These results indicate that CDCA1-LPs-specific IFN-γ production is derived from CDCA1-LPs-specific $CD4^+$ T-cells. Interestingly, specific response to CDCA1-LPs were induced or augmented by repeated vaccinations in some HNC patients (Fig. 5*e*). These observations suggest APCs collect and process a CDCA1 antigen derived from tumor cells killed by vaccine-induced CTLs and activate CDCA1-specific Th cells *in vivo*.

Discussion

Several phase I/II clinical trials using CDCA1-$A24_{56-64}$ SP against different types of tumor are ongoing. From these trials, we have observed that vaccination with a CDCA1-derived SP induces a CDCA1-specific CTL response and yields promising results in advanced cancer patients (Yoshitake *et al.*, manuscript in preparation). Therefore, we have attempted to identify CDCA1-LPs that induce antigen-specific Th1 cells and CTLs in order to further develop peptide vaccine immunotherapy.

In this study, we identified two immunogenic CDCA1-LPs encompassing both Th cell epitopes and CTL-epitopes. These CDCA1-specific CTLs were induced by efficient cross-presentation of CDCA1-LPs *in vitro* and *in vivo*. We demonstrated that the presence of CDCA1-LPs-specific Th cell responses in HNC patients, while there was no response in healthy donors after 1-week *in vitro* stimulation of PBMCs with CDCA1-LPs. CDCA1-LPs-specific responses in some HNC patients were augmented or induced by repeated CDCA1-$A24_{56-64}$ SP vaccination. Coulie and colleagues.[35,38,39] have examined the CTL repertoire of a melanoma patient showing complete regression of cutaneous metastases after MAGE-A3 vaccination with canary pox viral vector in combination with peptide. They showed that a new wave of tumor-specific CTL clones become detectable in the blood after vaccination and provided convincing evidence that the phenomenon of epitope spreading is critical to the development of effective antitumor immunity. These results suggest that the interaction between anti-vaccine CTLs and the tumor facilitates stimulation of large numbers of antitumor CTLs that proceed to destroy the tumor cells. We speculate that CDCA1-LP-specific Th cell responses in vaccinated HNC patients may be a phenomenon of intramolecular epitope spreading triggered by CTL-epitope vaccination.

Jandus *et al.*[40] reported that vaccination with a CTL-epitope peptide derived from Melan-A leads to not only the induction of antigen-specific CTLs but also drive the shift from a $Foxp3^+$ HLA-class II-restricted $CD4^+$ T-cells population to a Th1-like specific response. They examined the antigen-specific Th cells in metastatic melanoma patients vaccinated with a CTL-epitope peptide with *ex vivo* HLA-DQ6(*DQB1*06:02*)/Melan-A peptide multimer labeling. They showed the same Melan-A-derived CTL-epitope can be efficiently recognized by both HLA-DQ6-restricted Th cells and HLA-A2-restricted CTLs. We showed CDCA1-LP-specific Th cells generated from healthy donors did not respond to the CDCA1-$A24_{56-64}$ SP embedded in CDCA1-LPs. However, we did not evaluate whether CDCA1-specific $CD4^+$ T-cells derived from HNC patients vaccinated with CDCA1-$A24_{56-64}$ SP recognize the same sequence, CDCA1-$A24_{56-64}$ SP. In this regard, we need for more detailed analysis of circulating CDCA1-LP-specific $CD4^+$ T-cells in HNC patients.

Interestingly, T-cells obtained from HNC29 (HLA-$DR9^+$), HNC26 (HLA-$DR4^+$/$DR15^+$) and HNC35 (HLA-$DR4^+$) did not show any inhibition of responses to CDCA1-LPs in IFN-γ ELISPOT assay when the cultured cells were incubated with anti-DR mAb, but they showed marked inhibition when incubated with anti-DP or DQ mAb (Fig. 5*d*). We consider that CDCA1-LPs can encompass some HLA-class II-restricted T-cell epitopes which have not been identified by *in vitro* experiments in this study and can cover many cancer patients.

In this study, 74 and 68% of HNC patients demonstrated immunity to $CDCA1_{39-64}$-LP and $CDCA1_{55-78}$-LP respectively, and these are significantly higher than the population of healthy donors responding to CDCA1-LPs. Godet *et al.*[41] reported a possible synergistic effect of the telomerase-specific $CD4^+$ T-cell response with chemotherapy in lung cancer. They demonstrated that the existence of spontaneous telomerase-specific Th cells prior to first-line chemotherapy significantly increased overall survival in lung cancer that responds to chemotherapy. In addition, the recent introduction of immunotherapy in clinical practice emphasized the influence of immune responses on cancer prognosis and chemotherapy effectiveness.[9,10,42] These pieces of evidence support the hypothesis that induction or augmentation of CDCA1-specific Th cells by vaccination with CDCA1-LPs may improve the clinical outcome of cancer patients when combined with chemotherapy or other standard therapies.[43–45] Weide *et al.*[46] have recently reported that the presence of circulating Th cells responding to Melan-A or NY-ESO-1 has a strong independent prognostic impact on survival among chemotherapy-treated advanced melanoma patients. Thus, CDCA1-LPs-specific Th cell responses in HNC patients may positively influence overall survival. The impact of CDCA1-specific Th cell responses on clinical outcome will be evaluated in a future study.

Recent studies have shown that LPs vaccines encompassing CTL-epitopes are superior to those composed of minimal CTL-epitopes in anti-tumor CTL immunity because of long-lasting cross-presentation of the LPs.[8,47,48] Disis *et al.*[49] reported vaccination with HER-2/neu-derived LP which encompasses HLA-A2-restricted CTL-epitope elicited the embedded epitope-specific CTLs in breast cancer patients. We showed that cross-presentation of CDCA1-LPs induced priming and expansion of CDCA1-specific CTLs *in vitro* and *in vivo*. In addition, CDCA1-LPs encompass both Th cell epitopes and CTL-epitopes. Via cross-priming, tumor-specific $CD8^+$ T-cells can be elicited. Vaccination with CDCA1-LPs may therefore potentially elicit combined $CD4^+$ and $CD8^+$ T-cell responses. Such T-cell responses are considered to be crucial for tumor eradiation and generating long-term memory.[5] Finally, antigen-specific $CD4^+$ T-cells can enhance and sustain tumor-specific T-cell immunity over time. Our data also demonstrated that vaccination with CDCA1-LPs encompassing a CDCA1-$A24_{56-64}$ CTL-epitope was superior to CDCA1-$A24_{56-64}$ SP, as it elicits stronger effector CTL responses in HLA-A24 Tgm. However, we did not compare the capacity to induce CDCA1-specific CTLs between CDCA1-LPs and CDCA1-deived SPs in humans. This will be evaluated in a future study.

Although tumor antigen-specific $CD4^+$ T-cells promote the expansion and functions of $CD8^+$ T-cells *in vitro* and *in vivo*, MAGE-A3-derived helper peptide have been shown to induce MAGE-A3-specific HLA-DP4-restricted $CD4^+$ $Foxp3^+$ regulatory T-cells (Treg) after vaccination.[50] The number of Treg and CDCA1-specific Treg were not evaluated in this study, but these issues will be evaluated by ongoing monitoring before and after vaccination in a future study.

In conclusion, CDCA1-LPs encompassing CTL-epitopes may provide a useful tool for propagation of CDCA1-specific Th cells and CTLs. We present the first clinical evaluation of CDCA1-LPs-specific Th cell responses in HNC patients before and after CDCA1-derived CTL-epitope vaccination. These findings support a clinical trial of CDCA1 peptide-based immunotherapy for various types of cancers.

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Short Article

2. ヒトiPS細胞からの樹状細胞作製法の進歩

千住 覚

現在，がん免疫療法に用いられる樹状細胞は，患者自身から採取した末梢血単球を材料として作製されている．そして，白血球採取に伴う患者の身体的負担，採取できる細胞数や分化能力の個人差，高額の費用といった問題がある．われわれは，これらの問題を解決することを目的として，ヒトiPS細胞から樹状細胞を作製する分化誘導法を開発した．しかしながら，この方法は，分化誘導に時間と手間がかかり，実用性に乏しいと考えられた．そこで，樹状細胞の作製効率を改善するために，ヒトiPS細胞から増殖能力を有するミエロイド系血液細胞（iPS-ML）を作製する方法を開発した．

はじめに：がんに対する樹状細胞を用いた免疫療法

がんに対する能動免疫法の1つとして，樹状細胞療法が国内外において広く施行されている．免疫療法に用いられる樹状細胞は，通常，患者自身の末梢血中の単球を分化させることにより作製されている．単球を増殖させることはできないため，治療に必要な数の樹状細胞を調製するためには大量の単球を必要とする．そこで，末梢血白血球をアフェレーシス（成分採血）装置を用いて採取し，さらに単球を分離し樹状細胞に分化させるという方法がとられている．しかしながら，末梢血中の単球の数や樹状細胞への分化能力には，細胞ドナー間で個人差があり，がん患者の場合，しばしば，十分な数の樹状細胞が調製できない場合がある．さらに，患者個別のアフェレーシスと人手による培養操作が必要であるために費用が高額であることも問題である．このような問題を解決するためには，樹状細

[キーワード＆略語]
iPS細胞，樹状細胞，がん，iPS-ML，HLA

CTL：cytotoxic T lymphocyte
（細胞傷害性T細胞）
HLA：human leukocyte antigen
（ヒト白血球抗原）
iPS-DC：iPS cell-derived dendritic cell
（iPS細胞由来樹状細胞）
iPS-ML：iPS cell-derived myeloid cell line
（iPS細胞由来ミエロイド細胞ライン）
iPS-ML-DC：iPS-ML-derived dendritic cell
（iPS-ML由来樹状細胞）
iPS細胞：induced pluripotent stem cell
（人工多能性幹細胞）
TAP：transporter associated with antigen processing

Progress in dendritic cell generation from human iPS cells

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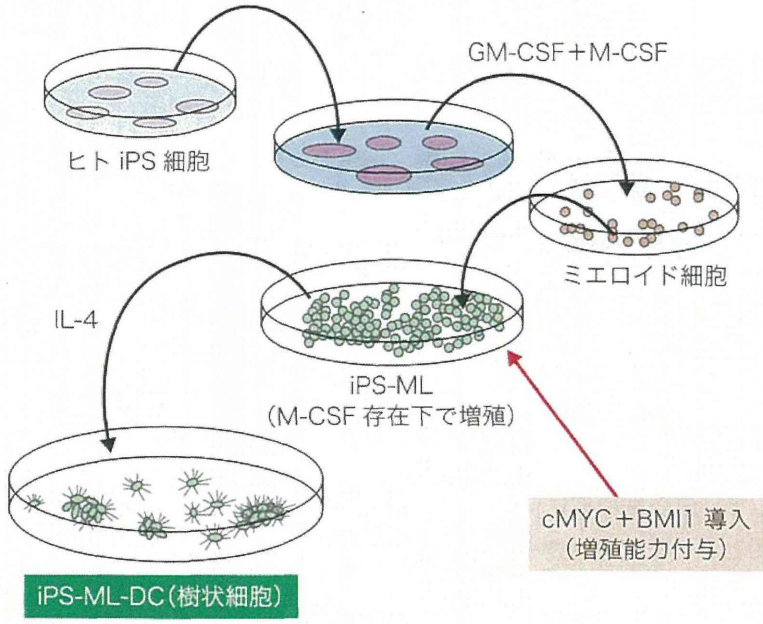

図1　iPS-ML および iPS-ML-DC の作製
ヒトの iPS 細胞から，増殖能力を有するミエロイド細胞（iPS-ML）を作製することができる．iPS-ML を分化させることにより樹状細胞（iPS-ML-DC）を作製することができる

胞を安定して，かつ，少ない費用で供給する手法を開発することが必要である．

1 iPS 細胞に由来する増殖性ミエロイド細胞（iPS-ML）

われわれは，前記のような課題を解決する目的で，以前より，多能性幹細胞に由来する樹状細胞による免疫療法の実現を目標として研究を行ってきた[1)〜5)]．2007年のヒト iPS 細胞の作製の発表を受け，iPS 細胞から樹状細胞を作製する研究を開始した．そして，2009年にマウスの，2011年にヒトの iPS 細胞からの樹状細胞作製を報告し，これを iPS-DC と名付けた[6)7)]．iPS-DC は単球由来の樹状細胞と同様に抗原提示能力や T 細胞刺激能力などを有しており，樹状細胞療法に使用することが可能であると考えられた．しかしながら，ヒト iPS 細胞を分化誘導して iPS-DC を作製するには1カ月以上の培養を必要とし，かつ，産生される iPS-DC の収量は分化誘導に使用した iPS 細胞の10〜20倍程度であった．このため，iPS-DC を作製して治療に使用することを想定すると，単球由来樹状細胞の場合よりもかえってコストがかかってしまうことが予想された．そこで，細胞産生効率を改善するべく試行錯誤を行った結果，樹状細胞の前駆細胞を大量に作製するという方法に到達した．この方法は，ヒト iPS 細胞由来のミエロイド系血液（$CD11b^+$）細胞に cMYC＋BMI1 などを導入することにより，増殖性を有するミエロイド系細胞，iPS-ML（iPS cell-derived myeloid cell line）を作製するものである（**図1**）．iPS-ML は，GM-CSF と M-CSF を含む培養液中で，倍加時間1〜2日で長期間にわたって増殖するので，得られる細胞数はほぼ無限である．

2 iPS-ML 由来の樹状細胞（iPS-ML-DC）とその問題点

iPS-ML は，IL-4 を添加して培養すると樹状細胞（iPS-ML-DC）へ分化する（**図1**）．iPS-ML-DC は，単球由来の樹状細胞と同様に強力な T 細胞刺激活性と細胞傷害性 T 細胞（CTL）誘導能力を有しており，細胞ワクチンとして使用可能であると考えられる．

樹状細胞の製造法として，iPS-ML を製造してストックしておき，必要に応じてこれを解凍し IL-4 を添加して2〜3日培養して ML-DC へ分化させるというシステムを構築すれば，樹状細胞を低コストで供給することが可能となると考えられた．しかしながら，患者自身の体細胞から iPS 細胞を樹立し，これを用いて iPS-ML および iPS-ML-DC を作製するやり方には，以

下のような問題がある．

① 患者個別にiPS細胞の樹立とiPS-ML作製を行う場合，相当の費用がかかることが予想され，コスト面から治療法としての実用化・普及は困難と考えられる．

② iPS細胞の樹立からiPS-ML，iPS-ML-DCの作製までを行うには2カ月以上の期間を必要とする．早急に治療を開始する必要がある場合には時間的に間に合わない．

③ iPS-MLからiPS-ML-DCへ分化した段階で増殖は停止するので，患者にiPS-ML-DCを投与した後，体内で増殖することはない．しかしながら，増殖性を有するiPS-MLの混入が絶対にないとは言い切れず，患者自身と同一の遺伝的背景のiPS-ML-DCを治療に用いた場合，その一部が腫瘍化（白血病化）する危険性がある．

3 iPS細胞ストックの利用とHLAタイプ別細胞供給システム（iPS-MLストック）

前述した問題を解決するためには，アロ（同種異系）のiPS-MLを事前に作製しておき，必要に応じてこれを使用できるシステムを構築することが必要である．iPS-MLはiPS細胞のもととなった体細胞のドナーに由来する遺伝的背景を有している．iPS-ML-DCをHLA型の異なる患者の治療に使用した場合には，強力なアロ免疫応答のため，投与したiPS-ML-DCはすぐに破壊されてしまうであろう．しかし，HLAが適合していれば，急性期のアロ免疫反応は弱くなり，拒絶反応のタイミングが遅くなるので，一定時間患者体内で生存できるため，腫瘍抗原特異的な免疫応答を誘導するための時間を確保できる．現在，iPS細胞を用いた再生医療の実現に資する目的で，京都大学iPS細胞研究所において，日本人集団において頻度の高いHLAタイプのホモ接合ドナーに由来するiPS細胞ストックの設立が計画されている．このiPS細胞ストック由来のiPS細胞からiPS-MLを作製し，“iPS-MLストック”を構築しておくことが非常に有用であると考えられる（図2）．

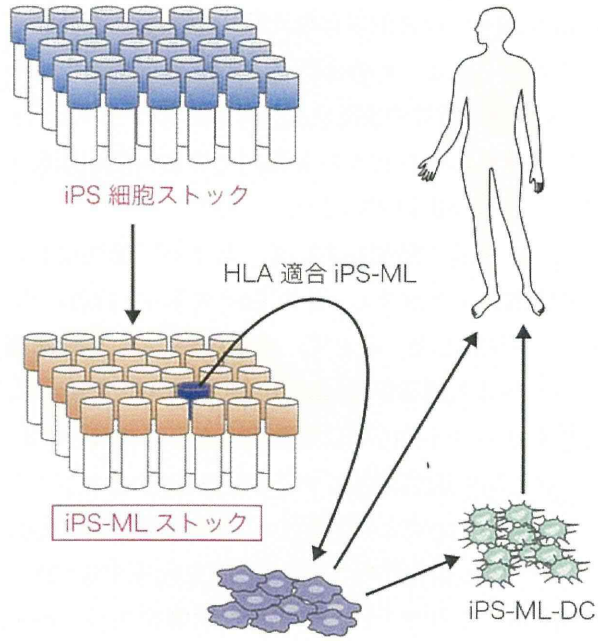

図2　iPS-MLストック
京都大学iPS細胞研究所において，日本人集団において頻度の高いHLAハプロタイプのホモ接合ドナーに由来するiPS細胞によるiPS細胞ストックの設立が計画されている．われわれは，iPS細胞ストックをベースとして“iPS-MLストック”を構築することを計画している

4 低頻度HLAタイプへの対応（TAP欠損iPS-ML）

iPS細胞ストックの問題点として，頻度の低いHLAハプロタイプに関しては，ホモ接合のドナーの確保が困難であると予想され，日本人の10～20％位はiPS細胞ストックでカバーできない可能性がある[8]．そこで，低頻度HLAハプロタイプの患者の治療にも対応できるiPS-MLを作製するため，ヒトiPS細胞においてHLA関連遺伝子の標的破壊を行って組織不適合性の問題を回避する手法を検討した．

HLAの一致しないアロ個体間で細胞移入を行った際，早期に発生する急性拒絶反応においては，不適合HLAクラスⅠを認識するCTLが主要なエフェクター細胞となる．HLAクラスⅠは，抗原ペプチドとの複合体として細胞表面に発現するが，HLAクラスⅠ/抗原ペプチド複合体の形成には，ペプチドトランスポーターであるTAP（transporter associated with antigen processing）の機能が必須である．TAPを欠損すると，細胞質において産生されたペプチドを小胞体内腔へ輸送することができないため，HLAクラスⅠ/抗原ペプチド複合体の形成が妨げられ，HLAクラスⅠが細胞表面で安定して発現することができない．このため，TAP欠損細胞では，細胞表面のHLAクラスⅠの発現が抑制

されるため，アロの個体に移入後の急性拒絶を回避できると考えられる．われわれは，TAPを欠損したマウスのES細胞に由来する樹状細胞を用いて，この仮説を検証し，アロのレシピエント体内での生存期間延長が可能という結果を得ている[9]．

さらに，最近の研究において，ヒトiPS細胞においてTAP2遺伝子を破壊し，そのTAP欠損iPS細胞から，iPS-MLを作製した．そして，本来HLA-A*02:01を有しないTAP2欠損iPS-MLにHLA-A*02:01を導入し，この細胞からiPS-ML-DCを作製した．このiPS-ML-DCに悪性黒色腫関連抗原であるMART1のペプチドをパルスしたものを刺激細胞として用いることにより，HLA-A*02:01を有するアロのドナーに由来する$CD8^+$T細胞から，MART1に特異的な細胞傷害性T細胞（HLA-A*02:01/MART1特異的なCTL）を誘導することができた[10]．この結果は，TAP欠損iPS-MLにさまざまなHLAクラスIを導入して“TAP欠損iPS-MLストック”を作製することにより，多様なHLA型に対応可能であることを示唆する．

おわりに

HLAが完全に一致した細胞であっても，多様なマイナー抗原（mHA）が拒絶抗原として働くため，免疫抑制剤の投与などにより免疫抑制状態が持続するような状況でない限り，アロの移植細胞が長期生着することはない．したがって，iPS細胞由来のHLA適合iPS-ML-DCを患者に投与しても，投与後に患者体内で増殖し腫瘍化することはない．TAPが欠損したアロのiPS-ML-DCも，同様に，腫瘍化する危険性はないと考えられる．しかしながら，腫瘍化の問題は，iPS細胞の安全性という観点からは最も懸念されるところであり，臨床試験を開始する前には，動物モデルを用いた長期の安全性確認を行う必要がある．

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Analysis of cytotoxic T lymphocytes from a patient with hepatocellular carcinoma who showed a clinical response to vaccination with a glypican-3-derived peptide

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Abstract. Glypican-3 (GPC3), which is a carcinoembryonic antigen, is overexpressed in human hepatocellular carcinoma (HCC). Previously, we performed a phase I clinical trial of GPC3-derived peptide vaccination in patients with advanced HCC, and reported that GPC3 peptide vaccination is safe and has clinical efficacy. Moreover, we proposed that a peptide-specific CTL response is a predictive marker of overall survival in patients with HCC who receive peptide vaccination. In this study, we established GPC3-derived peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who was vaccinated with an HLA-A2-restricted GPC3 peptide vaccine and showed a clinical response in the phase I clinical trial. Established CTL clones were analyzed using the IFN-γ ELISPOT assay and a cytotoxicity assay. GPC3 peptide-specific CTL clones were established successfully from the PBMCs of the patient. One CTL clone showed cytotoxicity against cancer cell lines that expressed endogenously the GPC3 peptide. The results suggest that CTLs have high avidity, and that natural antigen-specific killing activity against tumor cells can be induced in a patient with HCC who shows a clinical response to vaccination with the $GPC3_{144-152}$ peptide.

Introduction

Primary liver cancer, which is frequently hepatocellular carcinoma (HCC), is the sixth most common cancer and third most frequent cause of cancer-related death worldwide, and it is becoming more prevalent not only in East Asia, South-East Asia, and Africa but also in Western countries (1-3). Recently, the multikinase inhibitor sorafenib was demonstrated to prolong overall survival (OS) in patients with advanced HCC, and it has become the standard drug for first-line systemic treatment (4-6). However, based on the Response Evaluation Criteria in Solid Tumors (RECIST), the response rate for sorafenib is rather low, and the incidence of adverse events is relatively high, especially in elderly patients (7). Therefore, the generation of a novel effective therapy for HCC is a priority.

Immunotherapy is an attractive option for treating HCC. Many of the tumor antigens associated with HCC are potential candidates for peptide vaccines (8,9). The carcinoembryonic antigen Glypican-3 (GPC3), which is a 65-kDa protein of 580 amino acids, belongs to the family of glycosyl-phosphatidylinositol (GPI)-anchored heparan sulfate proteoglycans (HSPG) (10,11). GPC3 is specifically overexpressed in HCC (72-81% of cases) and correlates with poor prognosis (12-16). This suggests that GPC3 is an ideal target for anti-HCC immunotherapy.

We have previously demonstrated the antigenicity of GPC3, and that the HLA-A*24:02-restricted $GPC3_{298-306}$ (EYILSLEEL) peptide and the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide can induce GPC3-reactive CTLs without inducing autoimmunity (17-21).

HLA-A2 is the most frequent HLA-A type in all ethnic groups (22). HLA-A2 is also expressed in about 40% of Japanese persons (23,24) and in about 50% of Caucasians (25). Among Caucasians, >90% of HLA-A2-positive individuals carry the HLA-A*02:01 allele (25), whereas among the Japanese, there are multiple common and well-documented (CWD) allelic variants, including HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 (26). The frequencies of the HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07 alleles in the Japanese population are 19, 14 and 7%, respectively (26). Therefore, we confirmed that the HLA-A*02:01-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptide could also bind to HLA-A*02:06 and HLA-A*02:07 using a binding assay (unpublished data).

On the basis of these results, we conducted a phase I clinical trial of a GPC3-derived peptide vaccine in 33 patients with advanced HCC. The HLA-A*24:02-restricted $GPC3_{298-306}$ peptide was used for HLA-A*24:02-positive patients and

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Key words: glypican-3, peptide vaccine, CTL clone

the HLA-A*02:01-restricted $GPC3_{144-152}$ peptide was used for HLA-A*02:01, HLA-A*02:06 and HLA-A*02:07-positive patients. We found that GPC3 vaccination was well-tolerated, and that the GPC3 peptide vaccine induced a GPC3-specific CTL response in almost all of the patients (27-30). Moreover, the vaccination-induced GPC3-specific CTL response correlated with overall survival (OS); the OS was significantly longer in patients with high GPC3-specific CTL frequencies than in those with low GPC3-specific CTL frequencies (27). In terms of clinical responses, one patient showed a partial response (PR) and 19 patients showed stable disease 2 months after initiation of treatment. One patient with HCC who showed a PR was HLA-A*02:07-positive. In addition, several HLA-A*02:01-restricted GPC3 peptide-specific CTL clones with cytotoxic activities against GPC3 were established from the peripheral blood mononuclear cells (PBMCs) of patients vaccinated in this trial (27).

The aims of the present study were: i) to establish GPC3-derived, peptide-specific CTL clones from the PBMCs of an HLA-A*02:07-positive patient with HCC who showed a PR in the phase I clinical trial; and ii) to analyze the functions of these CTL clones.

Materials and methods

Ethics information. This study was approved by the Ethics Committee of the National Cancer Center and conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All the patients gave written informed consent before entering the study at the National Cancer Center Hospital East (Chiba, Japan). The trial has been registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR no. 000001395).

PBMCs collection. Peripheral blood samples were obtained pre- and post-vaccination from the patient with HCC who was HLA-A*02:07-positive. Post-vaccination, blood samples were collected from the patient every 2 weeks. The GMP-grade peptide $GPC3_{144-152}$ (FVGEFFTDV) (American Peptide Co., Sunnyvale, CA, USA) was emulsified in IFA (Montanide ISA-51 VG; SEPPIC, Paris, France) and injected intradermally at 30 mg/body three times at 14-day intervals (27,28). PBMCs were isolated by density centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and frozen in liquid nitrogen until use.

Cell lines. The human lung cancer cell line 1-87 ($GPC3^-$, HLA-A*02:07$^+$/A*11:01$^+$) and hepatitis B virus (HBV)-integrated human hepatocellular carcinoma cell line JHH-7 ($GPC3^+$, HLA-A*24:02$^+$/A*31:01$^+$) were conserved in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Company, St. Louis, MO, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA).

Plasmids and transfection. The expression vectors pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and pcDNA3.1 that contained the HLA-A*02:07 cDNA were used for the transfection experiments. The pcDNA3.1 construct that contained HLA-A*02:07 was kindly provided by Dr Ryo Abe (Tokyo University of Science, Chiba, Japan). The JHH-7/HLA-A*02:07 cell line was obtained by transfection of JHH-7 cells with the expression vector using FuGENE HD (Roche Applied Science, Mannheim, Germany). JHH-7/mock and JHH-7/HLA-A*02:07 cells were cultured in DMEM that was supplemented with 10% heat-inactivated FBS and 1 mg/ml G418 (Calbiochem, Darmstadt, Germany).

Induction of $GPC3_{144-152}$ peptide-specific CTLs from PBMCs. The PBMCs were cultured ($2x10^6$ cells/well) with the $GPC3_{144-152}$ peptide in RPMI-1640 (Sigma Chemical Company) that was supplemented with 10% heat-inactivated FBS, 100 IU/ml recombinant human IL-2 (Nipro, Osaka, Japan), and 10 ng/ml recombinant human IL-15 (PeproTech Inc, Rocky Hill, NJ, USA) for 14 days.

CD107a staining and flow cytometry analysis. $CD8^+$ T cells were isolated using human CD8 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from PBMCs that were stimulated with the $GPC3_{144-152}$ peptide for 14 days. The $CD8^+$ T cells were incubated with $GPC3_{144-152}$-pulsed or HIV_{19-27}-pulsed 1-87 cells at a ratio of 2:1 for 3.5 h at 37°C. CD107a-specific antibodies (BD Biosciences, San Jose, CA, USA) were included in the mixture during the incubation period.

Generation of CTL clones. $CD8^+$ $CD107a^+$ cells were sorted using a FACSAria cell sorter (BD Biosciences). Sorted CTLs were stimulated and the CTL clones were established as previously described (28).

Cytotoxicity assay. Cytotoxic capacity was analyzed with the Terascan VPC system (Minerva Tech, Tokyo, Japan). The CTL clone was used as the effector cell type. Target cells were labeled in calcein-AM 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 as previously described (28).

IFN-γ ELISPOT assay. Specific secretion of IFN-γ from human CTLs in response to stimulator cells was assayed using the IFN-γ ELISPOT kit (BD Biosciences), according to the manufacturer's instructions. Stimulator cells were pulsed with or without peptide for 1.5 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).

Determination of recognition efficiency. Calcein-AM-labeled target cells were pulsed with various concentrations of peptide, starting at 10^{-6} M and decreasing in log steps to 10^{-14} M. The CTL clones were incubated with the target cells at an effector:target (E/T) ratio of 10:1 for 4 h. The recognition efficiencies of the CTL clones were defined as previously described (28).

RNA interference. Human GPC3-specific siRNAs were chemically synthesized as double-strand RNA (Invitrogen). A non-silencing siRNA, AllStras Neg. Control siRNA, was obtained from Qiagen (Valencia, CA, USA). The following

GPC3-specific siRNA sequences were used: GPC3-siRNA (#4149), 5'-UUAUCAUUCCAUCACCAGAGCCUCC-3'; GPC3-siRNA (#4150), 5'-GGAGGCUCUGGUGAUGGAAU GAUAA-3': and GPC3-siRNA (#4151), 5'-UAUAGAUGACUG GAAACAGGCUGUC-3'. Synthetic siRNA duplexes were transfected using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocols.

RT-PCR. Using the TRIzol reagent (Invitrogen), we extracted total cellular RNA from untreated or siRNA (GPC3-siRNA or negative-siRNA)-treated JHH-7/HLA-A*02:07. cDNA was synthesized using the PrimeScript II 1st Strand cDNA Synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. The cDNA was added to a reaction mix that contained 10X Ex Taq Buffer (Takara), 2.5 mM dNTP mixture (Takara), 5 units Ex Taq (Takara), and 10 μM of the *GPC3*- or *β-actin*-specific PCR primers. The following primer sequences (sense and antisense, respectively) were used: for *GPC3*, 5'-AGCCAAAAGGCAGCAAGGAA-3' and 5'-AAGA AGAAGCACACCACCGA-3'; and for *β-actin*, 5'-CCTCGCCT TTGCCGATCC-3' and 5'-GGATCTTCATGAGGTAGTC AGTC-3'. PCR was performed using the 96-well Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA). PCR was performed for 20 cycles of 98°C for 10 sec, 64°C for 30 sec and 72°C for 30 sec, followed by a step of 72°C for 10 sec.

Sequence analysis of TCR-β gene. Using the TRIzol reagent (Invitrogen), total cellular RNA was extracted from established CTL clones. The cDNA of the *TCR-β* gene was synthesized using the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions, with the modification that we used 200 nM of the primer specific for the TCR-β chain constant region. The cDNA products were subjected to 2-step PCR, as previously described by Yukie Tanaka-Harada (35,36), and the PCR products were purified and sequenced in the Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems). The *TCR-β* variable (*TRBV*) gene, *TCR-β* joining (*TRBJ*) gene, *TCR-β* diversity (*TRBD*) alleles, and complementarity-determining region 3 (*CDR3*) sequences were identified using the IMGT databases (http://www.imgt.org/).

Results

$GPC3_{144-152}$ peptide-specific CTLs in the peripheral blood of the patient exert a clinical effect. We analyzed the immune responses of the patient who showed a PR following $GPC3_{144-152}$ peptide vaccination. In this patient, the supraclavicular lymph node metastases markedly regressed, two liver tumors disappeared, and the thoracic bone metastasis showed necrosis after the third vaccination (27). The levels of DCP decreased in the patients over the 2-month period. We evaluated the $GPC3_{144-152}$-specific immune responses in the peripheral blood using the *ex vivo* IFN-γ ELISPOT assay. For the HLA-A*02:07-positive patient with advanced HCC, the number and area of the spots increased after two rounds of vaccination, as compared with the pre-vaccination values, and the peak values were noted 10 weeks after the start of the treatment (Fig. 1A).

Establishment of $GPC3_{144-152}$-specific CTL clones from the PBMCs of the patient. To investigate the ability of the $GPC3_{144-152}$-specific CTLs induced by peptide vaccination to recognize antigen, we established CTL clones from the PBMCs of this patient 10 weeks after the start of treatment. The PBMCs were stimulated with the $GPC3_{144-152}$ peptide *in vitro* for 14 days. $CD8^+$ T cells were isolated from the stimulated PBMCs, and then incubated with peptide-pulsed 1-87 cells. $CD8^+$ $CD107a^+$ cells that reacted with the $GPC3_{144-152}$-pulsed 1-87 cells were sorted to the single-cell level. Thus, we established $GPC3_{144-152}$ peptide-specific CTL clones.

Three established CTL clones were analyzed for function using the IFN-γ ELISPOT assay and cytotoxicity assay. All of the CTL clones released IFN-γ in response to the $GPC3_{144-152}$-pulsed 1-87 cells, but not in response to non-pulsed 1-87 cells (Fig. 1B). Moreover, these CTL clones showed cytotoxicity against $GPC3_{144-152}$-pulsed 1-87 cells, but not against non-pulsed or HIV19-27-pulsed 1-87 cells (Fig. 1C). These results indicate that the CTL clones 24-4-2, 24-4-7 and 24-2-10 have specificity for the $GPC3_{144-152}$ peptide.

Functional avidity of the $GPC3_{144-152}$-specific CTL clones. We evaluated the cytotoxicity profiles of the CTL clones for 1-87 cells pulsed with a decreasing concentration series (from 10^{-6} to 10^{-14} M) of the $GPC3_{144-152}$ peptide. The peptide concentration at which the curve reached 50% cytotoxicity was defined as the recognition efficiency of the clone. The recognition efficiencies of CTL clones 24-4-2, 24-4-7 and 24-2-10 were 10^{-11}, 10^{-9} and 10^{-8} M, respectively (Fig. 2). This result suggests that CTL clone 24-4-2 has a higher avidity than the other two clones and, conversely, that CTL clone 24-2-10 has a lower avidity than the other two clones.

A $GPC3_{144-152}$-specific CTL clone recognizes cancer cells that endogenously express GPC3. Next, we tested the reactivities of these CTL clones against cancer cell lines that expressed GPC3 and HLA-A*02:07. We used the JHH-7/mock ($GPC3^+$, HLA-A*02:07-) and JHH-7/HLA-A*02:07 ($GPC3^+$, HLA-A*02:07$^+$) transfectants as the target cells (Fig. 3A). The CTL clone 24-4-2 (with high avidity) produced IFN-γ and was cytotoxic for JHH-7/HLA-A*02:07 cells but not for JHH-7/mock cells (Fig. 3B and C). The other clones did not produce IFN-γ and did not exhibit cytotoxicity for the two target cell lines. These results suggest that only high-avidity CTLs recognize cancer cells that express GPC3 peptide endogenously.

CTL clone 24-4-2 shows specificity for GPC3. To ascertain the GPC3 antigen-specific response of CTL clone 24-4-2, we created a GPC3 knockdown via siRNA treatment of the JHH-7/HLA-A*02:07 cells. GPC3 expression by the JHH-7/HLA-A*02:07 cells was clearly decreased by the GPC3-siRNA, as assessed by RT-PCR (Fig. 4A). We examined the IFN-γ production levels of CTL clone 24-4-2 against JHH-7/HLA-A*02:07 cells treated with GPC3-siRNA. IFN-γ production by CTL clone 24-4-2 was significantly decreased by the GPC3-siRNA (Fig. 4B). These results indicate that the HLA-A2-restricted $GPC3_{144-152}$ peptide is processed naturally by cancer cells, and that both HLA-A*02:07 and HLA-A*02:01 can present the $GPC3_{144-152}$ peptide on the surfaces of cancer cells.

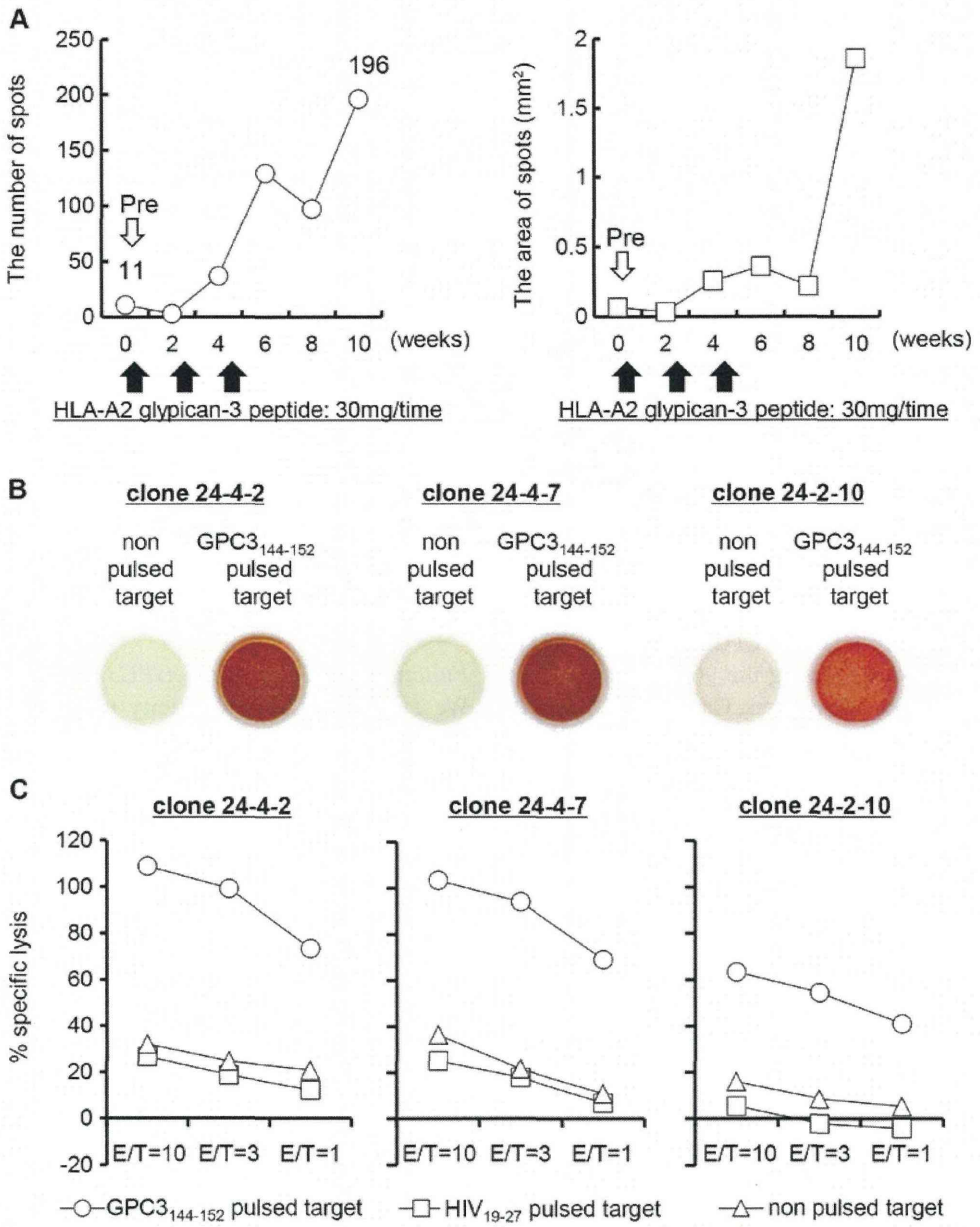

Figure 1. GPC3 peptide-specific CTL clones established from the PBMCs of a patient following GPC3 peptide vaccination. (A) Changes in the frequencies of $GPC3_{144\text{-}152}$ peptide-specific CTLs before and after vaccination in a patient who showed a PR post-vaccination. Changes in the GPC3 peptide-specific CTLs are observed as differences in the number (left) and the area (right) of spots in an *ex vivo* IFN-γ ELISPOT assay. (B) Results of the IFN-γ ELISPOT assay against peptide-pulsed target. HLA-A*02:07$^+$ cancer cell line 1-87 was used as the target. The target was pulsed with the $GPC3_{144\text{-}152}$ peptide. A non-pulsed target was used as the negative control. The ratio of effector cells to target cells (E/T) is 1. (C) Results of the cytotoxicity assay against peptide-pulsed target. The 1-87 cells were used as the target. Non-pulsed and $HIV_{19\text{-}27}$ peptide-pulsed targets were used as negative controls. E/Ts are 10, 3 and 1, respectively. A representative of three experiments is shown.

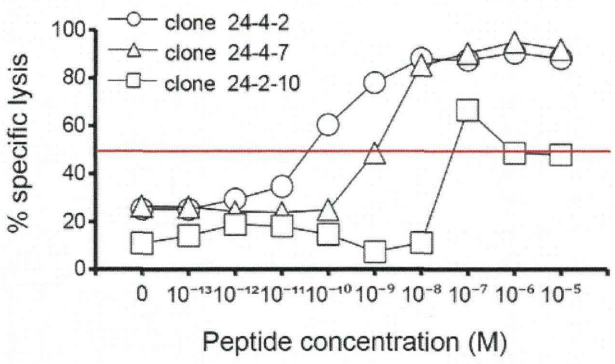

Figure 2. $GPC3_{144\text{-}152}$ peptide-specific avidity of the established CTL clones. The established CTL clones were tested for avidity using 1-87 cells that were pulsed with various concentrations of the $GPC3_{144\text{-}152}$ peptide. The peptide concentration at which the curve crossed the 50% cytotoxicity mark was defined as the recognition efficiency of that clone. E/T is 10. A representative of three experiments is shown.

Established CTL clones have different sets of TCR-β alleles. We analyzed the *TCR-β* gene sequences of the established CTL clones. The *TRBV*, *TRBJ* and *TRBD* alleles were identified using the IMGT databases. Thus, we identified the *TRBV*, *TRBD* and *TRBJ* alleles of the CTL clones (Table I). Each of the established CTL clones had different allele sets.

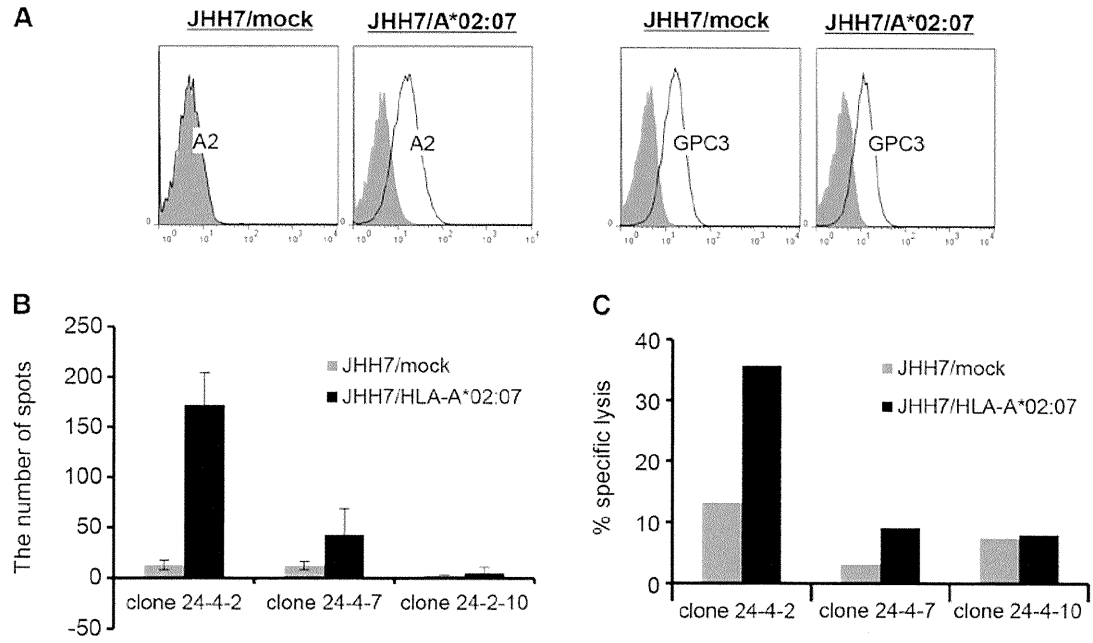

Figure 3. Recognition of GPC3$^+$ cancer cells by the established CTL clones. (A) Expression of HLA-A2 (left panel) and GPC3 (right panel) on established GPC3$^+$ HLA-A*02:07$^+$ cancer cells and control cells. (B) Results of the IFN-γ ELISPOT assay for the GPC3$^+$ cancer cell line. The HLA-A*02:07-overexpressing GPC3$^+$ cancer cell line, JHH7/HLA-A*02:07, was established and used as the target. JHH7/mock cells were used as the negative control. E/T ratio, 1. Data are presented as mean ± SD of three independent batches. (C) Results of the assay for cytotoxicity against the GPC3$^+$ cancer cell line. JHH7/HLA-A*02:07 cells were used as the target. JHH7/mock cells were used as the negative control. E/T is 3. A representative of three experiments is shown.

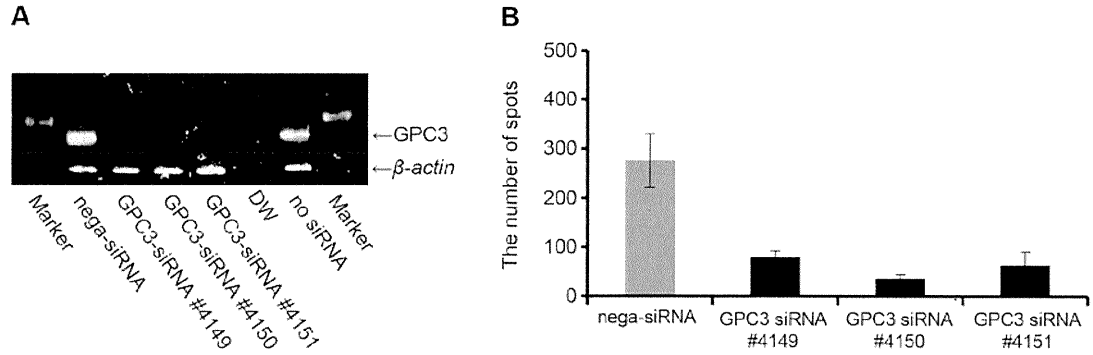

Figure 4. GPC3 specificity of CTL clone 24-4-2. (A) GPC3 expression levels on JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or negative (nega)-siRNA for 48 h, as determined by RT-PCR. (B) Results of the IFN-γ ELISPOT assay for JHH7/HLA-A*02:07 cells treated with GPC3-siRNA or nega-siRNA. E/T is 1. Data are presented as mean ± SD of three independent batches.

*CTL clone 24-4-2 is subject to HLA-A*02:07 restriction.* We investigated whether CTL clone 24-4-2 recognized the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex and the $GPC3_{144-152}$ peptide-HLA-A*02:06 complex, as well as the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex. Healthy donor PBMCs with HLA-A*02:01, HLA-A*02:06, HLA-A*02:07 and HLA-A*24:02 were used as the targets, and an HLA-A*02:01-restricted, GPC3-specific CTL clone, which is a previously established CTL clone (26), was used as the control. The HLA-A*02:01-restricted CTL clone recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:01 complex, and CTL clone 24-4-2 recognized only the $GPC3_{144-152}$ peptide-HLA-A*02:07 complex (Fig. 5). These outcomes indicate that CTL clone 24-4-2 has HLA-A*02:07 restriction.

Table I. TCR-β chain sequencing for established CTL clones.

No.	TRBV	TRBJ	TRBD
Clone 24-4-2	18*01	1-2*01	1*01
Clone 24-4-7	7-3*01	2-7*01	1*01
Clone 24-2-10	7-6*01	2-1*01	2*01

Discussion

Clinical trials of peptide-based vaccines are underway in several parts of the world. However, the monitoring of individual CTL post-vaccination has scarcely been reported in

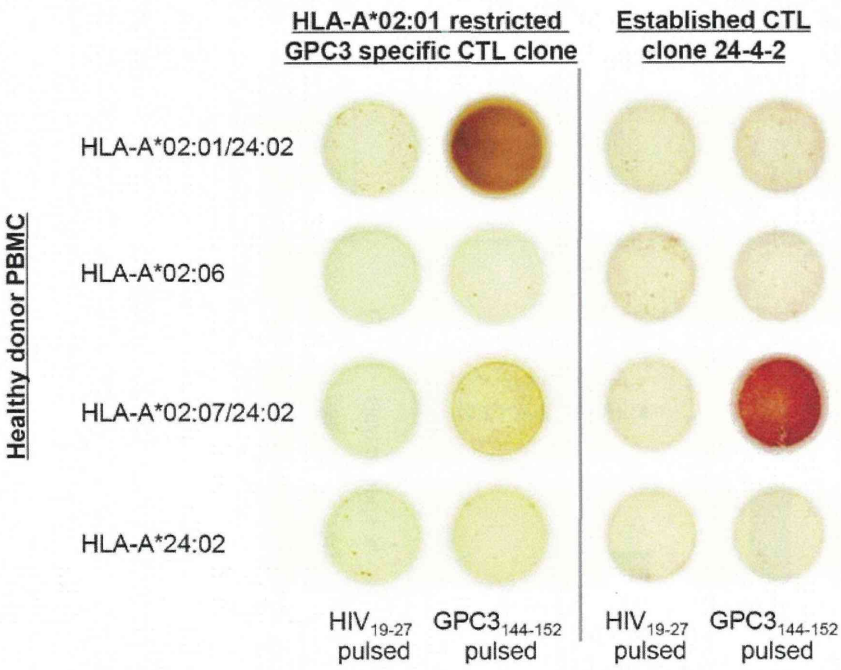

Figure 5. CTL clone 24-4-2 shows HLA-A*02:07 restriction. Results of the IFN-γ ELISPOT assay for healthy donor PBMCs with HLA-A2. The established CTL clone 24-4-2 and the HLA-A*02:01-restricted, GPC3-specific CTL clone were used as effectors. E/T is 0.2. A representative of two experiments is shown.

immunotherapy trials. In the present study, we established HLA-A*02:07$^+$ $GPC3_{144-152}$-specific CTL clones from the PBMCs of a patient who showed a PR following GPC3-derived peptide vaccination and we performed functional analyses against established CTL clones.

This patient showed an increase in the number of CTLs specific for the GPC3-derived peptide in the peripheral blood after vaccination (Fig. 1A) (27,28). Ten weeks after the start of treatment, the $GPC3_{144-152}$-specific CTL counts had increased approximately 18-fold, as compared with the pre-vaccination counts. In this case, analysis of the established CTL clones after vaccination could lend support to the notion that the vaccine-induced CTLs exert an antitumor effect, since few $GPC3_{144-152}$-specific CTLs were detected before vaccination.

In the present study, we confirmed that $GPC3_{144-152}$-specific CTL clones are cytotoxic for both $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that express GPC3 peptide endogenously. Confirming that the GPC3 peptide-specific CTL clones kill cancer cells that express endogenously the antigen peptide is important because antigen-derived and CTL-inducible peptides are not necessarily presented by cancer cells that endogenously express the antigen (31-33). Three established CTL clones showed cytotoxic activities related to their avidity for $GPC3_{144-152}$-pulsed 1-87 cells and JHH-7/HLA-A*02:07 cells that expressed the GPC3 peptide endogenously. These results show that although CTLs with different avidity can be isolated, only those CTLs with high avidity can kill cancer cells that express the antigen peptide endogenously. Several investigators have demonstrated a correlation between T-cell avidity and target recognition by T-cell populations that recognize murine tumor models and human cancers (34). Our results strongly support this observation.

The TCR usage of antigen-specific T cells is thought to be influenced by the affinity of the TCR for the antigen peptide-HLA class I complex. Several studies on the TCR usage of tumor-associated antigen (TAA)-specific T cells have used the *TRBV* gene family (35-41). These studies mainly analyzed the frequencies of TAA tetramer positive CD8$^+$ T cells. Although it is important to examine quantitative aspects, such as the frequencies of TAA tetramer positive CD8$^+$ T cells, the cytotoxicity of these T cells against cancer cells that express the TAA peptide endogenously cannot be confirmed. Moreover, GPC3 dextramer positive CD8$^+$ T cells were not detected in the PBMCs of the patients with HCC before GPC3 peptide vaccination (27,28). To analyze biased usage of the *TCR* gene of GPC3 dextramer positive CD8$^+$ T cells in the patients with HCC before and after GPC3 peptide vaccination, a new detection system with greater sensitivity *ex vivo* will be required. In the present study, we analyzed the *TCR-β* genes of the established $GPC3_{144-152}$-specific CTL clones, to confirm that these CTL clones have different TCRs. Our experiments show that the established CTL clones have different TCR-β-chain allele sets, i.e., *TRBV*, *TRBD* and *TRBJ* alleles (Table I), and different CDR3 sequences (data not shown). These results suggest that various GPC3-specific CTLs are induced by $GPC3_{144-152}$ peptide vaccination.

A*HLA-A*02:07 differs from HLA-A*02:01 by a single non-conservative change (Y to C) at residue 99. X-ray crystallographic data have identified position 99 as one of the residues forming the D secondary pocket, which engages the residue at position 3 on peptide ligands (42-44). Although hHLA-A*02:07 was originally not included in the HLA-A2 supertype, cross-reactivity between HLA-A*02:07 and other A2 subtypes was detected at the functional level (44,45). Moreover, this HLA molecule indeed binds a subset of the peptide repertoire bound by other A2 subtypes (44). For these reasons, HLA-A*02:07 should also be included in the A2 supertype (46). Ito *et al* (47) and Nonaka *et al* (48) reported that an HLA-A2-restricted

CTL line established from the tumor-infiltrating lymphocytes (TIL) of an HLA-A*02:07-positive patient showed significant cytotoxicities for HLA-A*02:01-, HLA-A*02:06- and HLA-A*02:07-positive cancer cells. Therefore, we examined whether the $GPC3_{144-152}$-specific CTL clone 24-4-2, which was established from the PBMCs of an HLA-A*02:07- positive patient with HCC, could recognize HLA A-A*02:01 or HLA-A*02:06. However, this CTL clone failed to recognize HLA-A*02:01 or HLA-A*02:06.

We have reported previously on the detection via immunohistochemical staining of massive infiltration of CD8-positive T cells into the remaining liver tumor of this patient (27). It was difficult to confirm that these tumor-infiltrating $CD8^+$ T cells have specificity for GPC3. Currently, we are conducting clinical testing of liver biopsies taken before and after GPC3 peptide vaccination of patients with advanced HCC. Our aim is to reveal the GPC3 peptide-specific immune responses induced by the GPC3-derived peptide vaccine in both the peripheral blood and the tumor. We are analyzing the *TCR* gene sequences of CD8 or GPC3 dextramer positive T cells in both the peripheral blood and tumor. Already in this trial, a remarkable clinical effect has been observed for an HLA-A*02:07-positive patient with HCC who received $GPC3_{144-152}$ peptide vaccination (49).

HLA-A*02:07 is present in the populations of East Asia, South-East Asia (7%), and northern India (11.5%) (26,50-52). In southern China, the frequency of the HLA-A*02:07 allele is reported to be even higher than the frequency of the HLA-A*02:01 allele (53,54). In addition, about 75% of liver cancer cases occur in South-East Asia, including China, Hong Kong, Taiwan, Korea, India and Japan (55). Taking together these previous reports and our results, it appears that HLA-A*02:07-positive patients with HCC are good candidates for $GPC3_{144-152}$ peptide vaccination. Further studies will be necessary to prove the clinical efficacy of GPC3 peptide vaccination for advanced HCC.

In conclusion, we present substantial evidence that $GPC3_{144-152}$-specific CTLs with different TCR allele sets that are induced in patients with HCC who show a PR following $GPC3_{144-152}$ peptide vaccination indicate not only high avidity but also natural antigen-specific killing activity against tumor cells.

Acknowledgements

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Identification of HLA-A2 or HLA-A24-restricted CTL epitopes for potential HSP105-targeted immunotherapy in colorectal cancer

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Abstract. We previously reported that heat shock protein 105 (HSP105) is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer and has proven to be a novel biomarker for the immunohistochemical detection of these cancers. In the present study, we used HLA-transgenic mice (Tgm) and the peripheral blood mononuclear cells (PBMCs) of colorectal cancer patients to identify HLA-A2 and HLA-A24-restricted HSP105 epitopes, as a means of expanding the application of HSP105-based immunotherapy to HLA-A2- or HLA-A24-positive cancer patients. In addition, we investigated by *ex vivo* IFN-γ ELISPOT assay whether the HSP105-derived peptide of cytotoxic T cells (CTLs) exists in PBMCs of pre-surgical colorectal cancer patients. We found that four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYKQDL) and HSP105 A24-7 (EYVYEFRDKL), are potential HLA-A2 or HLA-A24-restricted CTL HSP105-derived epitopes. HSP105-specific IFN-γ-secreting T cells were detected in 14 of 21 pre-surgical patients with colorectal cancer in response to stimulation with these four peptides. Our study raises the possibility that these HSP105 peptides are applicable to cancer immunotherapy in patients with HSP105-expressing cancer, particularly colorectal cancer.

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Introduction

Colorectal cancer is one of the most prevalent cancers and a major cause of mortality worldwide (1). Although adjuvant systemic chemotherapy or chemoradiation can confer a limited but significant survival advantage, novel and more effective therapies are needed. To improve survival rates, new therapeutic agents have been investigated. Immunotherapy for colorectal cancer is a promising candidate treatment, and there is evidence that host immune responses can influence survival (2). Ideal targets for immunotherapy are gene products overexpressed in cancer cells but silenced in normal tissues, with the exception of immune-privileged tissues, such as that of the testis.

We previously reported that heat shock protein 105 (HSP105), identified by SEREX, is overexpressed in a variety of human cancers, including colorectal, pancreatic and esophageal cancer, but with little to no expression in normal tissues aside from the testis (3,4). HSP105 is a stress protein induced by various stressors and belongs to the HSP105/110 family and plays an important role as a chaperone under physiological conditions (5). Using immunohistochemical analysis, we previously found that HSP105 was specifically overexpressed in 44 of 53 (83.0%) colorectal cancer patients (4). It has also been reported that DNA vaccination with both HSP105 and bone marrow-derived dendritic cells (BM-DCs) pulsed with HSP105 led to tumor rejection of colorectal cancer but did not induce an autoimmune reaction in mice (6-8).

This suggests that HSP105 presents a useful tumor-specific antigen target for immunotherapy. However, HSP105-derived epitope peptides of $CD8^+$ T cells have not been identified. The gene frequency of HLA-A24 (A*24:02) is relatively high in Asian populations, especially the Japanese, but low in Caucasians. On the other hand, the gene frequency of HLA-A2 (A*02:01) is high among several ethnic groups, including Asians and Caucasians (9). Therefore, HLA-A2 or HLA-A24-restricted cytotoxic T cell (CTL) HSP105 epitopes could be extremely

useful for immunotherapy in a large portion of patients worldwide. In the present study, we identified human HSP105-derived CTL epitopes restricted by HLA-A2 or HLA-A24 using HLA-transgenic mice (Tgm) and examined whether these epitope-based peptides could activate HSP105-reactive CTLs in peripheral blood mononuclear cells (PBMCs) of patients with colorectal cancer.

Materials and methods

Mice. HLA-A2.1 (HHD) Tgm, H-2D$^{b-/-}$β2m$^{-/-}$ double-knockout mice introduced with the human β2m-HLA-A2.1(α1 α2)-H-2D^{b} (α3 transmembrane cytoplasmic) (HHD) mono-chain gene construct were generated in the Departmente SIDA-Retrovirus, Unite d' Immunite Cellulaire Antivirale, Institut Pasteur, Paris, France (10,11) and were kindly provided by Dr F.A. Lemonier. HLA-A24.2 (HHD) Tgm were purchased from Japan SLC, Inc. (Shizuoka, Japan). Female 6- to 8-week-old BALB/c mice (H-2K^{d}) and BALB/c nude mice, purchased from Charles River Japan (Yokohama, Japan), were maintained and handled in accordance with animal care policy.

Cell lines. The human colorectal cancer cell line SW620 (endogenously expressing HSP105 and HLA-A*02:01, 24:02) and human liver cancer cell line HepG2 (HSP105-low expressing and HLA-A*02:01, 24:02), were kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). Murine colorectal cancer cells, Colon26 (C26) (endogenously expressing HSP105 and H-2K^{d}) were kindly provided by Dr Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). T2 cells (a TAP-deficient and HLA-A*02:01-positive cell line) were provided by Kyogo Ito of Kurume University. Cells were maintained *in vitro* in RPMI-1640 or DMEM supplemented with 10% FCS.

RNA interference. Small interfering RNAs targeting human HSP105 were chemically synthesized by Dharmacon Research (HSP105-siRNA and luciferase; Lafayette, CO, USA) as previously described (12), with the following siRNA sequences: HSP105-siRNA, UUGGCUGCAACUCCGAUU GTT and luciferase, CGUACGCGGAAUACUUCGATT. The transfection of siRNA oligonucleotides was carried out using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines.

Peptides. Human HSP105-derived peptides, identical in amino acid sequence with mouse HSP105 and expressing the binding motifs for HLA-A*02:01- and HLA-A*24:02-encoded molecules, were designed with BIMAS software (BioInformatics and Molecular Analysis Section; Center for Information Technology, NIH, MD, USA). We purchased a total of 16 versions of peptides carrying the HLA-A2 (A*0201)-binding motifs and 9 versions of peptides carrying the HLA-A24 (A*2402)-binding motifs from Biologica (Tokyo, Japan) (Table I).

Induction of HSP105-reactive CTLs in Tgm. Peptide immunizations in mice were performed as previously described (13). In brief, bone marrow (BM) cells (2x10^{6}) from HLA-A2 or HLA-A24 Tgm were cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (5 ng/ml) and 2-mercaptoethanol (0.8 ng/ml) for 7 days in 10-cm plastic dishes. These BM-DCs were pulsed with the two HSP105 peptide mixtures (1 μmol/l each peptide) for 2 h at 37˚C. We primed the HLA-A2 or HLA-A24 Tgm with the syngeneic BM-DC vaccine (5x10^{5}/mice) into the peritoneal cavity twice, once per week. Seven days following the last immunization, the spleens were collected and CD4^{-} spleen cells were isolated by negative selection with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to exclude any nonspecific IFN-γ production from the CD4^{+} spleen cells co-cultured with the BM-DCs. The CD4^{-} spleen cells (2x10^{6}/well) were stimulated with syngeneic BM-DCs (2x10^{5}/well) that had been pulsed with each peptide *in vitro*. After 6 days, the frequency of cells producing IFN-γ/2x10^{4} CD4^{-} spleen cells upon stimulation with syngeneic BM-DCs (1x10^{4}/well), pulsed with or without each peptide, was assayed using an enzyme-linked immunospot (ELISPOT) assay as previously described (13).

Identification of a CTL epitope in BALB/c mice. The peptide immunizations in mice were performed as previously described (14). Splenocytes removed from mice 7 days following the last immunization were harvested and cultured in 24-well culture plates (2.5x10^{6}/well) in 45% RPMI, 45% AIMV, 10% FCS and supplemented with recombinant human interleukin 2 (100 U/ml), 2-mercaptoethanol (50 μmol/l) and each peptide (10 μmol/l). After 5 days, the cytotoxicity of these cells against target cells was assayed using standard 6-h ^{51}Cr release assays (15).

Blood samples. Blood samples from cancer patients were collected during routine diagnostic procedures after obtaining formal consent from patients at the Kumamoto University Hospital, from April to September 2006 and from patients at the National Cancer Center Hospital East, from December 2006 to March 2007. The study was approved by the local ethics committee, and informed consent was obtained from all patients.

Induction of HSP105-reactive human CTLs. We isolated PBMCs from heparinized blood of HLA-A24^{+} and/or HLA-A2^{+} Japanese patients with colorectal cancer using Ficoll-Conray density gradient centrifugation; peripheral monocyte-derived dendritic cells (DCs) were generated as previously described (16,17). CD8^{+} T cells were isolated with CD8 microbeads (Miltenyl Biotec, Bergisch Gladbach, Germany) from PBMCs of the same donor and peptide-reactive CD8^{+} CTLs were generated. Five days following the last stimulation, the cytotoxic activities of the CTLs against cancer cell lines were measured by ^{51}Cr-release assay as previously described (15). For these assays, CTLs were co-cultured with each cancer cell line, as the target cells (5x10^{3}/well), at the indicated effector/target ratio.

In vivo tumor challenge. Subcutaneous tumors were induced in mice by injecting 1x10^{4} SW620 cells suspended in 100 μl PBS or Hanks' balanced salt solution (Gibco, Grand Island, NY, USA) into the backs of BALB/c nude mice. Tumor incidence and volumes were assessed weekly using calipers and tumor areas were measured. Results are presented as mean tumor areas ± SD.