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Identification of novel human leukocyte antigen-A*11:01-restricted cytotoxic T-lymphocyte epitopes derived from osteosarcoma antigen papillomavirus binding factor

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Osteosarcoma is the most common malignancy of bone that affects young people. Neoadjuvant chemotherapy and surgery have significantly improved the prognosis. However, the prognosis of non-responders to chemotherapy is still poor. To develop peptide-based immunotherapy for osteosarcoma, we previously identified CTL epitopes derived from papillomavirus binding factor (PBF) in the context of human leukocyte antigen (HLA)-A2, HLA-A24 and HLA-B55. In the present study, we identified two novel CTL epitopes, QVT (QVTVWLLEQK) and LSA (LSALPPPLHK), in the context of HLA-A11 using a sequence of screenings based on the predicted affinity of peptides, in vitro folding ability of peptide/HLA-A11 complex, reactivity of peptide/HLA-A11 tetramer and interferon (IFN)-γ production of T cells that was induced by mixed lymphocyte peptide culture under a limiting dilution condition. CTL clones directed to QVT and LSA peptides showed specific cytotoxicity against HLA-A11$^+$PBF$^+$ osteosarcoma (HOS-A11) cells. In contrast, another epitope, ASV (ASVLSRRLGK), could highly induce cognate tetramer-positive CTL. This might be because the ASV peptide mimics the peptide ASV (R6Q) (ASVLSQRLGK) derived from bacterial polypeptides, ROK family proteins. However, ASV-induced CTL did not show cytokine production against the cognate peptide. In conclusion, the CTL epitopes QVT and LSA peptides might be useful for the development of immunotherapy targeting PBF for patients with osteosarcoma.

KEYWORDS
CTL epitope, in vitro folding ability, MHC-tetramer, osteosarcoma, PBF

1 | INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor of childhood and adolescence.[1] Current treatment for osteosarcoma includes neoadjuvant chemotherapy and surgical resection with an adequate margin followed by reconstruction.[2] This management strategy has dramatically improved the outcome and raised the 5-year survival rate to 60%-70% for patients with osteosarcoma. However, the prognosis for patients with advanced, metastatic, or recurrent osteosarcomas is still poor.[3] Therefore, the development of new therapeutic approaches is urgently needed.

In recent years, T cell-mediated immunotherapies have played an increasing role in cancer treatments including cancer vaccines, adoptive cell transfer and T-cell receptor (TCR)-modified T-cell

therapy.[4] To develop T cell-mediated immunotherapies for osteosarcoma, we previously identified papillomavirus binding factor (PBF) as an osteosarcoma-associated antigen recognized by an autologous CTL clone in the context of human leukocyte antigen (HLA)-B55.[5] Immunohistochemical analysis showed that 92% of biopsy specimens of osteosarcoma expressed PBF. Moreover, PBF-positive osteosarcoma had a significantly poorer prognosis than that with negative expression of PBF.[6] So far, CTL epitopes restricted by HLA-A24 and HLA-A2 have been identified by a reverse immunology approach and tetramer-based frequency analysis.[6,7]

Previous studies on CTL epitopes derived from tumor-associated antigens (TAA) mostly focused on HLA-A2 and HLA-A24 alleles because of the higher worldwide frequency of these alleles. However, HLA-A11 is also present at a high frequency in worldwide populations, particularly in South-East Asia. To date, 15 supertype alleles (HLA-A*11:01 to -A*11:15) have been reported with HLA-A*11:01 being the most prevalent. The most common HLA-A alleles and their frequencies in South-East Asia are HLA-A24 (0.242), HLA-A2 (0.226) and HLA-A11 (0.281) (http://www.allelefrequencies.net). Therefore, identification and characterization of PBF-specific CTL epitopes restricted by HLA-A11 alleles are extremely important to facilitate the development of PBF-targeted immunotherapies for osteosarcoma.

In the present study, we aimed to identify novel HLA-A*11:01-restricted CTL epitopes derived from PBF using a sequence of screenings based on the predicted affinity of peptides, in vitro folding ability of peptide/HLA-A11 complex, reactivity of peptide/HLA-A11 tetramer and production of interferon (IFN)-γ of T cells induced by mixed lymphocyte peptide culture under a limiting dilution condition. We also aimed to determine whether CTL clones reacting with each of the PBF peptides recognized naturally presented peptides on osteosarcoma cells.

2 | MATERIALS AND METHODS

The present study was carried out in accordance with the guidelines established by the Declaration of Helsinki and was approved by the institutional review board of Medical & Biological Laboratories Co., Ltd and Sapporo Medical University. Healthy donors provided informed consent for the use of blood samples in our research.

2.1 | Donors and cells

Peripheral blood samples were obtained from 5 HLA-A*11:01-positive healthy donors. DNA typing of the HLA-A loci was carried out by the PCR-SSOP-Luminex method using Genosearch HLA-A (MBL, Nagoya, Japan). The osteosarcoma cell line HOS was obtained from ATCC (Manassas, VA, USA). HOS cells expressed the PBF protein and maintained the HLA genotype as follows: A*02:11, B*52:01, C*12:02. For a cytotoxicity assay of HLA-A*11:01-restricted CTL epitopes, HOS cells were transfected with the pcDNA 3.1 vector (Thermo Fisher Scientific, Inc., Waltham, MA, USA) encoding cDNA for HLA-A*11:01, followed by selection using G418 (200 μg/mL). The stable transfectant was designated as HOS-A11. All cell culture reagents were purchased from Sigma-Aldrich (Tokyo, Japan) unless otherwise specified.

2.2 | Design and synthesis of PBF-derived peptides

Amino acid sequences of the PBF protein were screened with the HLA-peptide binding prediction software of NetMHC 3.4 (http://www.cbs.dtu.dk/services/NetMHC/) for oligomeric peptides potentially capable of binding to HLA-A11 molecules.[8] Nine candidate peptides with high scores were selected (Table 1). They were synthesized using automated solid phase techniques and purified by reversed-phase HPLC, and their sequences were verified by mass spectrometry. The HLA-A*11:01-binding peptide ATVQGQNLK derived from CMV pp65 protein and the HLA-A*24:02-binding peptide AYACNTSTL derived from survivin-2B were also synthesized and used as controls.[9] All peptides were obtained in lyophilized form, dissolved in DMSO at a concentration of 20 mg/mL, and stored in aliquots at −30°C until use. A stock solution of 1 mg/mL was prepared by further dilution in RPMI1640. The first three letters of the peptide sequences are used as shorthand identification throughout the article.

2.3 | In vitro folding of peptide-MHC complexes

As described previously,[10,11] the HLA-A*11:01 heavy chain and β2-microglobulin (β2m) were expressed as inclusion bodies in *Escherichia coli*. Then, the inclusion bodies were separately dissolved in a solution of 8 mol/L urea. To generate peptide-MHC (pMHC) complexes, the HLA-A*11:01 heavy chain was diluted more than 100-fold into a folding buffer containing β2m and a peptide and then incubated to reach steady-state pMHC formation. After incubation at 4°C for different periods, the folding solution was collected and analyzed by gel-filtration HPLC. Peak areas of pMHC complexes were calculated from the absorption wavelength at 280 nm. The in vitro folding abilities are represented by the areas of pMHC peaks.

2.4 | Mixed lymphocyte peptide culture under a limiting dilution condition

Peripheral blood mononuclear cells of HLA-A*11:01-positive healthy donors were subjected to mixed lymphocyte peptide culture under a limiting dilution condition according to the method described by Karanikas et al[12] with some modifications. PBMC were suspended at a cell density of 2×10^6 cells/mL. Then, PBMC were seeded at 2×10^5 cells/well into round-bottom 96-microwell plates with 100 μL RPMI1640 containing 2 mmol/L L-glutamine (Invitrogen, Grand Island, NY, USA), 55 μmol/L 2-mercaptoethanol (Invitrogen), 100 IU/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), and 5% (v/v) autologous plasma in the presence of each synthetic peptide (10 μg/mL). After 72 hours, an equal volume of a medium containing 100 U/mL interleukin 2 (recombinant human IL-2, S-6820; Shionogi

TABLE 1 Characteristics of papillomavirus binding factor-derived peptides with HLA-A*11:01 binding motif

Peptide	Sequence[a]	Position	Binding score[b]	Quantitative value of in vitro folding test (μV/s)[c]			Frequency of CTL precursor among $CD8^+$ cells					Effector function	
				Day 1	Day 3	Day 7	Donor A	Donor B	Donor C	Donor D	Donor E	IFN-γ	Cytotoxicity
ASV	A**S**VLSRRLG**K**	2-11	0.647	192 708	285 534	329 966	$\geq 1.25 \times 10^{-5}$	$\geq 1.39 \times 10^{-5}$	$\geq 1.43 \times 10^{-5}$	ND	ND	–	–
SVL	S**V**LSRRLG**K**	3-11	0.741	233 347	303 809	338 556	$\geq 1.25 \times 10^{-5}$	$\geq 1.39 \times 10^{-5}$	$\geq 1.43 \times 10^{-5}$	ND	ND	–	ND
QVA	Q**V**AFQPGQ**K**	72-80	0.483	220 083	306 752	337 567	–	–	–	–	–	ND	ND
QVT	Q**V**TVWLLEQ**K**	103-112	0.537	188 398	278 013	280 883	–	–	–	1.74×10^{-7}	1.58×10^{-7}	+	+
KVL	K**V**LRSIVGI**K**	289-298	0.585	213 275	293 307	325 019	–	–	–	–	–	ND	ND
LSA	L**S**ALPPPLH**K**	364-373	0.627	201 859	233 836	238 863	–	–	1.49×10^{-7}	ND	ND	+	+
SAL	S**A**LPPPLH**K**	365-373	0.696	220 695	317 418	332 346	–	–	–	ND	ND	ND	ND
SSL	S**S**LPSGALS**K**	386-395	0.752	219 122	310 843	339 146	–	–	–	–	–	ND	ND
SLP	S**L**PSGALS**K**	387-395	0.572	223 695	307 917	353 665	–	–	–	–	–	ND	ND
AYA[d]	AYACNTSTL	–	0.072	157 878	161 031	135 674							
ATV[e]	A**T**VQGQNL**K**	–	0.643	158 088	269 031	300 090							

Amino acid sequences of candidate peptides are listed in the order of position.

Frequency of CTL precursor was evaluated using the following calculation: (number of tetramer-positive wells)/([total number of tested wells] × [initial number of $CD8^+$ cells per well]).

HLA, human leukocyte antigen; IFN, interferon.

-, Not detected; ND, Not determined.

[a]Peptide sequences are given as the single-letter code for amino acids, and the predicted anchor residues are shown in bold.

[b]Binding score was determined by NetMHC 3.4 HLA peptide binding predictions.

[c]Ability of each peptide was evaluated by a developed in vitro folding system.

[d]Peptide derived from survivin-2B.

[e]Peptide derived from cytomegalovirus pp65.

Pharmaceutical Co., Osaka, Japan) was added to each well, and the medium was replaced every 2 or 3 days thereafter with a fresh medium containing IL-2 (50 U/mL). Tetramer staining was carried out on days 14-21. To establish CTL clones, tetramer-positive T cells were seeded in 96-well round-bottom microplates at 1 cell per well with irradiated allogeneic PBMC (5×10^4) in 100 μL RPMI1640 containing 10% autologous plasma and cognate peptide (100 ng/mL). The next day, IL-2 was added to a final concentration of 50 U/mL. Fourteen days after the stimulation, T cells in positive wells were tested by a tetramer assay.

2.5 | Major histocompatibility complex-tetramer production and staining

Peptide/MHC tetramers (MHC-tetramers) were produced as described previously.[10] Briefly, recombinant β2m and the extracellular portion of the HLA-A*11:01 heavy chain containing the BirA recognition sequence in frame at its C-terminus were expressed in *E. coli* as inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric peptide-MHC complexes were refolded around peptides by dilution denaturing conditions. After buffer exchange, a specific lysine residue in the heavy chain C-terminal tag was biotinylated with the BirA enzyme. Monomeric complexes were purified by monomeric avidin gel chromatography (Pierce, Rockford, IL, USA). Tetrameric arrays of biotinylated peptide-MHC complexes were formed by the addition of phycoerythrin (PE)-labeled streptavidin (ProZyme, Hayward, CA, USA) at a molar ratio of 4:1. MHC-tetramers are presented as the first three letters of their peptide (eg, ASV-tet for the HLA-A*11:01 ASVLSRRLGK tetramer). PBMC were stained with MHC-tetramers and FITC-conjugated anti-CD8 mAb (Beckman Coulter, Miami, FL, USA) at 4°C for 30 minutes. After washing twice, stained cells were resuspended in FACS buffer (PBS plus 2% FBS and 0.05% NaN_3) containing 0.5 μg/mL 7-AAD (Beckman Coulter) for dead cell exclusion before analysis. Cells were gated on 7-AAD-negative cell populations, and data were analyzed using FACSCalibur and CELLQuest software (Becton Dickinson, San Jose, CA, USA). Percentages of tetramer-positive cells were expressed relative to the total number of $CD8^+$ T cells. Frequency of tetramer-positive CTL was evaluated using the following calculation: (number of tetramer-positive wells)/([number of total tested wells] × [number of $CD8^+$ cells per well]).

2.6 | Intracellular IFN-γ staining

Intracellular cytokine staining was done as previously described with some modifications.[13] Cultured cells were restimulated by the same peptide at various concentrations (up to 10 μg/mL) for 15 hours in the presence of 10 μmol/L brefeldin A. Brefeldin A inhibits the export of proteins from the endoplasmic reticulum and results, therefore, in the intracellular accumulation of cytokines, which would otherwise be secreted. Cells were harvested, washed, and stained with phycoerythrin-Cy5 (PC5)-conjugated anti-CD8 mAb (Beckman Coulter) at room temperature for 15 minutes. The cells were simultaneously stained with MHC-tetramers as described above. After washing, the cells were fixed in PBS containing 2% formaldehyde for 15 minutes at room temperature, permeabilized in 0.1% saponin and 2% BSA in PBS, and stained with an FITC-labeled antibody reacting with human IFN-γ (Beckman Coulter). Samples were analyzed on FACSCalibur with CELLQuest software.

2.7 | Cytotoxicity assay

Cytotoxicity of tetramer-positive CTL clones was measured by FACSCalibur using an IMMUNOCYTO Cytotoxicity Detection Kit (MBL). Osteosarcoma cells (HOS or HOS-A11 cells) were used as the target cells. The target cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; Dojin, Kumamoto, Japan) at room temperature for 15 minutes. After washing three times, CFSE-labeled target cells were suspended in RPMI1640 and seeded in microwells (5×10^3 cells/100 μL per well). Tetramer-positive CTL clones stimulated with the QVT or LSA peptide by LD/MLPC (mixed lymphocyte peptide culture under a limiting dilution condition) were used as effector cells. Tetramer-positive CTL clones were suspended in 100 μL RPMI1640 and mixed with the labeled target cells at various effector/target ratios. After a 5-hour incubation period at 37°C, the specific killing activity of the CTL clones against each target was monitored by fluorescent Annexin V staining and calculated as ([experimental Annexin V-positive frequency of target cells − spontaneous Annexin V-positive frequency of target cells]/[100 − spontaneous Annexin V-positive frequency of target cells]) × 100%.

3 | RESULTS

3.1 | Ability of PBF-derived synthetic peptides to bind to HLA-A*11:01 molecules

To determine HLA-A*11:01-restricted epitopes of PBF, we synthesized nine peptides from the amino acid sequence of PBF in accordance with the NetMHC 3.4 scores for HLA-A*11:01 affinity (Table 1). Next, we investigated whether these potential peptides could form pMHC complexes by using a newly developed in vitro folding system. For in vitro folding, we expressed HLA-A*11:01 in *E. coli*, dissolved the inclusion bodies in urea buffer, and folded the denatured protein with human β2m and with each peptide. We then determined the binding ability of the peptide by measuring an amount of pMHC complex with a gel-filtration HPLC assay. In order to determine the utility of our folding system, a set of known epitopes (HLA-A*11:01-binding peptide ATVQGQNLK derived from CMV pp65 protein and HLA-A*24:02-binding peptide AYACNTSTL derived from survivin-2B) were used as a positive or negative control. The positive control peptide folded with HLA-A*11:01 molecules showed a significant increase in peak areas of pMHC complexes in a time-dependent method. In contrast, although an empty MHC peak could be observed, the negative

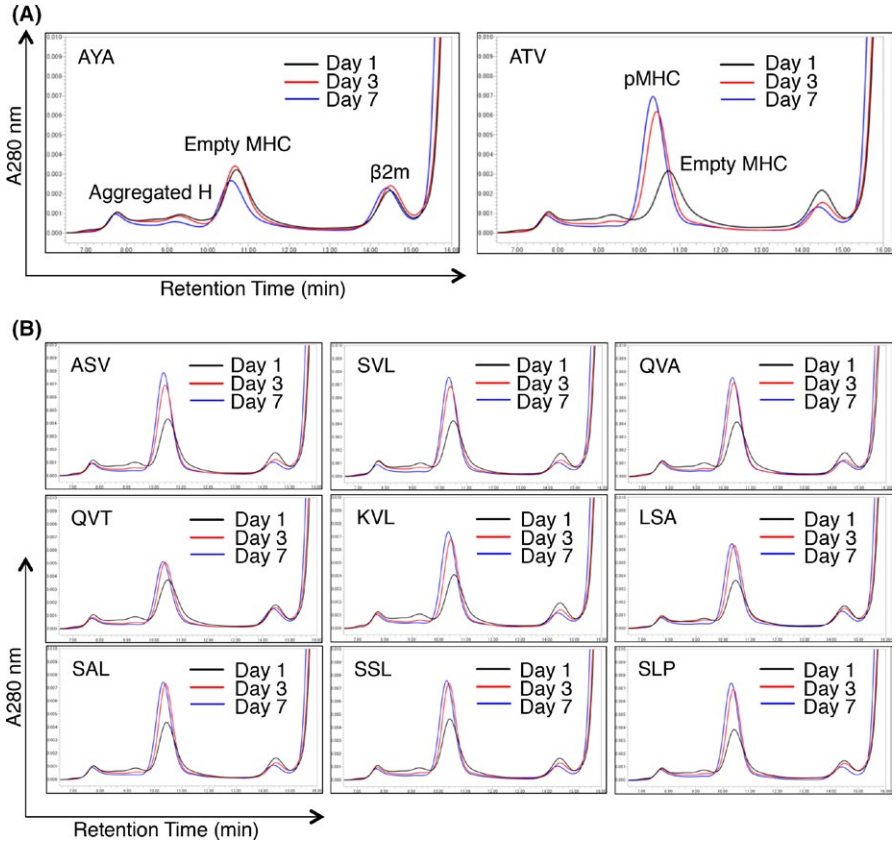


FIGURE 1 In vitro folding of papillomavirus binding factor (PBF)-derived peptides. Gel filtration chromatograms of the refolded products obtained. The aggregated H (heavy chain), the empty MHC, the correctly refolded peptide-MHC (pMHC) complex (10~11 min), and the extra β2m are indicated (A). The pMHC complexes on day 1 (in black), day 3 (in red), and day 7 (in blue) are shown (B)

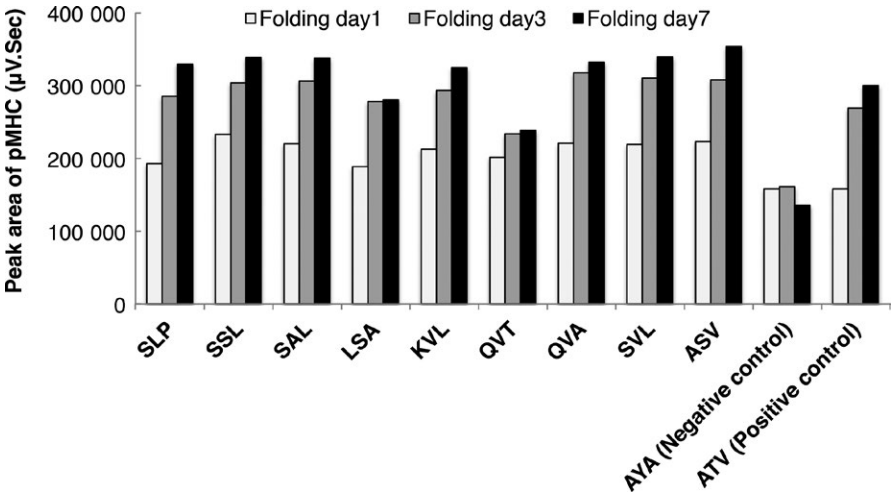


FIGURE 2 Peak areas of peptide/MHC complexes at different time periods. The peak areas were calculated from the absorption wavelength at 280 nm on day 1 (in white), day 3 (in gray), and day 7 (in black)

control (HLA-A*24:02-binding peptide) folded with HLA-A*11:01 molecules showed no obvious change in the peak areas regardless of the incubation period (Figure 1A). Because a larger pMHC complex peak indicates better peptide-MHC binding ability, the results indicate that our system is useful for determining the ability of potential peptides to bind to HLA-A*11:01 molecules. In vitro folding ability of these peptides was also correlated with the prediction score of NetMHC 3.4 (Table S1, Data S1).

For potential peptides of PBF, in vitro folding of each peptide was carried out as described above. All nine peptides that folded with HLA-A*11:01 molecules showed a substantial increase in pMHC complex peaks along with incubation time as well as the positive control peptide (Figure 1B, Table 1). Quantified peak areas are shown in Figure 2. Based on the results of in vitro folding, we confirmed that these potential peptides are true MHC binders to HLA-A*11:01 molecules.

3.2 | Identification of immunogenic CTL epitopes using a peptide/HLA-A11 tetramer

To determine whether these potential peptides can activate specific $CD8^+$ T cells in vitro, PBF-specific CTL were induced by mixed lymphocyte peptide culture (MLPC) under a limiting dilution condition. Fourteen days after peptide stimulation, cells were stained with a tetramer that was prepared from the cognate peptide. To set up a more rapid assay system for screening of CTL epitopes, we used three to five potential peptides as a peptide mixture to stimulate PBMC in the presence of IL-2 and autologous plasma but without additional antigen-presenting cells. After MLPC, the cells were stained with tetramers as follows: In the first step, an aliquot of cells from each of the eight wells in a column of a 96-well plate was pooled to make 12 pools (columns 1-12). Then, these samples were stained separately with mixed cognate tetramers. In the second step, pools yielding antigen-specific CTL were further tested for binding of mixed tetramers to cells from individual wells that contributed to the positive pool. In the third step, samples from positive wells were separately stained with individual tetramers to determine which peptide was the specific CTL epitope.

As shown in Figure 3, three peptides (QVA, LSA and SLP) were used for MLPC in Donors A, B and C. In the first step, 12 pools (columns 1-12) were stained with mixed tetramers (QVA-tet, LSA-tet and SLP-tet). A positive pool with 0.51% tetramer-positive cells was detected in column 10 of Donor C (Figure 3A). In the second step, mixed tetramer-positive cells were detected in G well (10-G well) (Figure 3B). In the third step, tetramer-positive cells were detected by LSA-tet but not by QVA-tet or SLP-tet (Figure 3C).

Next, five peptides (QVA, QVT, KVL, SSL and SLP) were used for MLPC in Donors D and E. As shown in Figure 4A, mixed tetramer-positive cells were detected in two pools (column 7 and column 2 in Donor D and Donor E, respectively) in the first step. Two mixed-tetramer-positive wells were detectable in 7-B and in 2-C wells in Donor D and Donor E, respectively, in the second step (Figure 4B). Only QVT-tet-positive cells were detected in both 7-B and 2-C wells in the third step (Figure 4C).

We then carried out MLPC using a mixture of three peptides (ASV, QVT and SAL). In contrast, a high percentage of tetramer-positive cells (up to 38% of $CD8^+$ cells) was detected in all columns of Donors A, B and C (Figure 5A). We tested the reactivity of each tetramer of column 7 in Donor A, column 4 in Donor B and column 4 in Donor C. As shown in Figure 5B, ASV-tet-positive cells were observed, suggesting that the ASV peptide might be a highly immunogenic CTL epitope.

We also tested another mixture (SVL, KVL and SSL). SVL-tet-positive cells were detected in Donors A, B and C (Figure 6), suggesting that immunogenic CTL epitopes derived from PBF in the context of HLA-A*11:01 might be LSA, QVT, ASV and SVL (Table 1).

3.3 | Cross-reactivity of CTL epitopes between 9-mer and 10-mer peptides

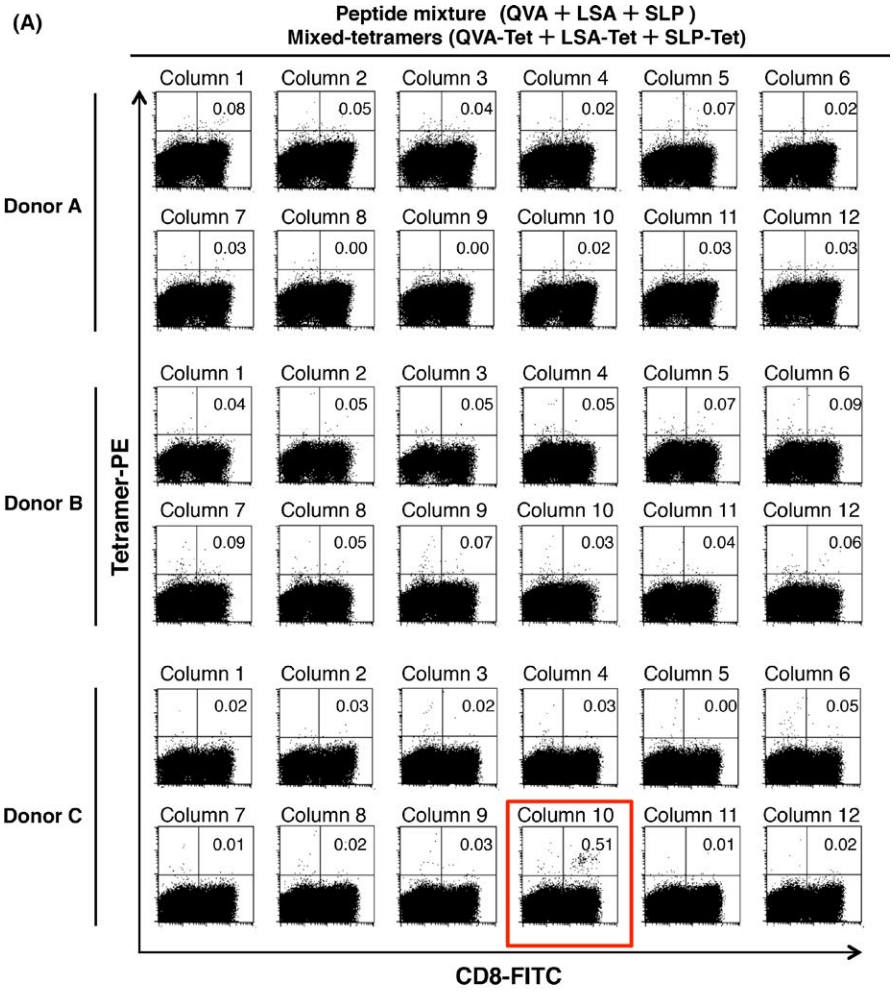
Both the 9-mer SVL (*SVLSRRLGK*) and the 10-mer ASV (A*SVLSRRLGK*) could induce peptide-specific CTLs in vitro (Figures 5, 6). Next, we investigated whether the two epitopes have some cross-reactivity with each other. As shown in Figure S1A, both ASV-induced and SVL-induced CTL positively reacted with both ASV-tet and SVL-tet. These results suggested that these two epitopes have cross-reactivities. Regarding peptide-specific CTL induction, 10-mer ASV might be more immunogenic than 9-mer SVL.

We also assessed cross-reactivity between 10-mer LSA (L*SALPPPLHK*) and a 9-mer SAL (*SALPPPLHK*). LSA-induced CTL reacted with LSA-tet but not with SAL-tet. In addition, LSA could not induce LSA-tet-positive cells (Figures 5B, S1B). No cross-reactivity was observed between the LSA and SAL peptides. The specific pMHC-TCR interaction suggests that 10-mer LSA is the optimal-length CTL epitope.

By comparison of ASV-induced CTL with LSA and QVT-induced CTL, a difference was observed in CTL precursor frequency (Table 1). As shown in Figures 3 and 4, only one tetramer-positive well was detected in a 96-well plate after MLPC using LSA and QVT. These results suggested low frequencies of peptide-specific CTL precursors in peripheral blood $CD8^+$ T cells (3×10^{-7}). It is well known that the frequency of tumor-associated antigen (TAA)- specific CTL precursors is below 10^{-6} in healthy peripheral blood.[6,12] To our surprise, tetramer-positive CTL after MLPC using ASV peptide were detected in all columns of Donor A, Donor B and Donor C (Figure 5). It is very likely that the probability of such an occurrence is driven by T-cell cross-reactivity as a result of amino acid sequence homology between ASV and other peptides derived from some foreign antigen. Based on these considerations, we carried out a protein-protein BLAST search for short exact matches on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/) to retrieve homology data across species. As a result, we identified a homologous peptide that has one amino acid substitution at position 6 of the 10-mer ASV epitope (ASVLSRRLGK→ASVLSQRLGK). The ASV (R6Q) peptide is derived from the ROK family protein (Pfam 00480), which is a large family of bacterial polypeptides that includes many functionally uncharacterized gene products.[14] We carried out MLPC to investigate whether the two epitopes have cross-reactivity with each other. As shown in Figure S1C, both PBF-ASV and ROS-ASV (R6Q) peptides could induce CTL that positively reacted with ASV-tet. ASV (R6Q)-tet could also react with CTL induced by both peptides, but the reactivity was lower than that of ASV-tet. These results suggest that PBF-ASV and ROS-ASV (R6Q) might share the TCR repertoire with different pMHC-TCR affinities.

3.4 | Cytokine production capacity of peptide-induced CTL

Next, we carried out intracellular IFN-γ staining to evaluate the functionality of CTL induced by QVT, LSA, ASV or SVL epitope. Resultant CTL after MLPC were restimulated with a cognate peptide at various concentrations in the presence of brefeldin A. As a result, both QVT and LSA efficiently induced IFN-γ production in a dose-dependent way (Figure 7A,B). In contrast, although CTL were



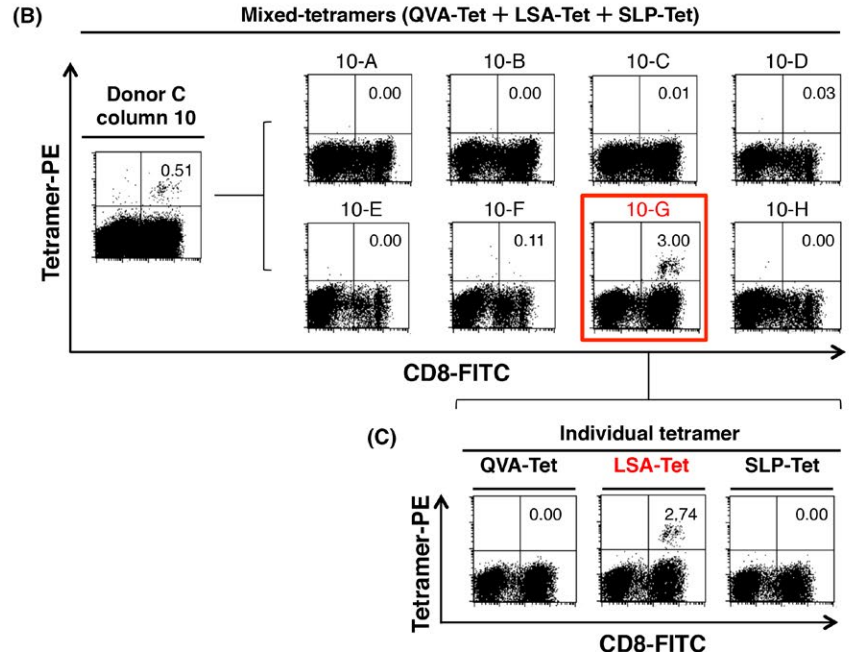


FIGURE 3 MHC-tetramer reactivity with CTL after mixed lymphocyte peptide culture using a mixture of QVA, LSA and SLP peptides. PBMC from three healthy subjects (Donors A, B and C) were stimulated with a peptide mixture (QVA, LSA and SLP). After peptide mixture stimulation, cells of columns 1-12 were stained with mixed tetramers (QVA-tet, LSA-tet and SLP-tet). A positive pool with 0.51% tetramer-positive cells was detected in column 10 of Donor C (A). Next, individual wells that contributed to the positive pool were further stained by mixed tetramers for each well (A-H). A positive well was detected in G well (10-G) with 3.00% tetramer-positive cells (B). Finally, cells from the positive well were separately stained with individual tetramers. CTL were detected with 2.74% LSA-tet-positive cells (C). Frequency of tetramer-positive cells is shown as the percentage of total $CD8^+$ T cells

detectable with MHC-tetramers, ASV and SVL could not induce specific IFN-γ production (Figure 7C,D). Peptide-specific tumor necrosis factor (TNF)-α production and expression of CD107a and CD137 were not observed (Figures S2, S3, Data S1). These results indicated that functional CTL could be induced by QVT and LSA but not by ASV and SVL.

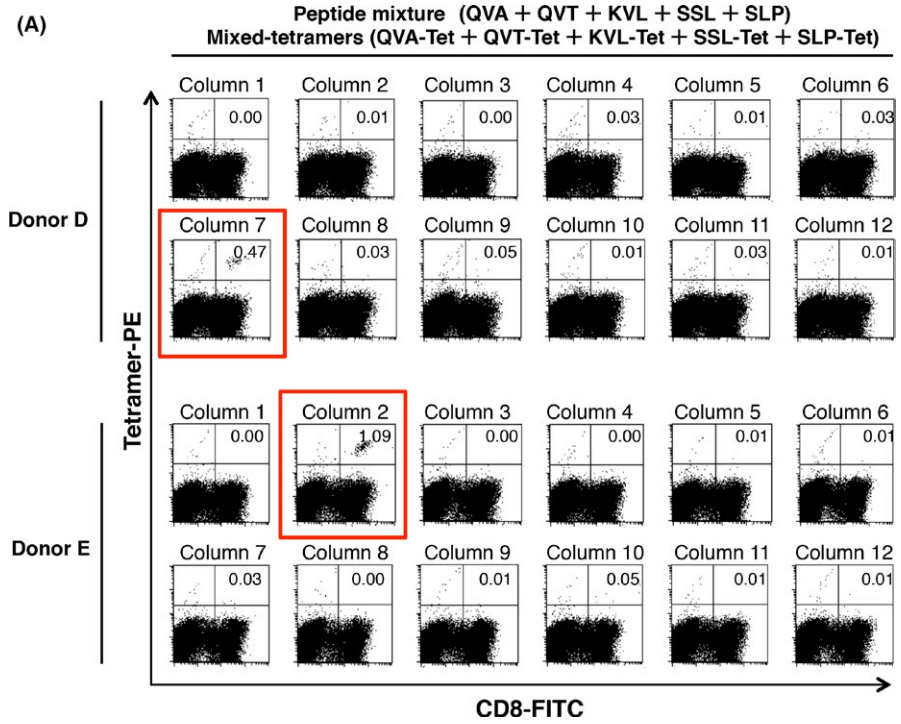


FIGURE 4 MHC-tetramer reactivity with CTL after mixed lymphocyte peptide culture using a mixture of QVA, QVT, KVL, SSL and SLP peptides. PBMC from two healthy subjects (Donors D and E) were stimulated with a mixture of peptides (QVA, QVT, KVL, SSL and SLP). After peptide mixture stimulation, cells of columns 1-12 were stained with mixed tetramers (QVA-tet, QVT-tet, KVL-tet, SSL-tet and SLP-tet). Two positive pools were detected in column 7 of Donor D and in column 2 of Donor E (A). Next, individual wells that contributed to the positive pool were further stained by mixed tetramers for each well (A-H). A positive well was detected in B well (7-B) of Donor D and in C well (2-B) of Donor E, separately (B). Finally, cells from the positive well were stained with individual tetramers. Only QVT-tet-positive CTL were detected (C). Frequency of tetramer-positive cells is shown as the percentage of total $CD8^+$ T cells

3.5 | QVT- and LSA-specific CTL clones recognized naturally presented peptides on osteosarcoma cells in the context of HLA-A11

Finally, we assessed the cytotoxic activity of tetramer-positive cells against osteosarcoma cell lines. Osteosarcoma cells (HOS and HOS-A11 transfectant cells) were used as the target cells. Tetramer-positive CTL clones stimulated with QVT or LSA were used as effector cells (designated as QVT CTL or LSA CTL). As depicted in Figure 8, QVT CTL and LSA CTL showed specific cytotoxicity against HOS-A11 in an effector : target ratio-dependent way. In contrast, none of the CTL clones showed cytotoxic activity against HOS (PBF-positive, HLA-A*11:01-negative). Silencing of PBF expression using siRNA inhibited VT CTL- and LSA CTL-mediated cytotoxicity against HOS-A11 cells (Figures S4, S5, Data S1). These results indicated that PBF was naturally processed and presented by HLA-A11 molecules on the surfaces of HOS-A11 cells. The cytotoxic activity of QVT CTL was stronger than that of LSA CTL. These results are consistent with the results of intracellular IFN-γ staining (Figure 7). In contrast, the ASV CTL clone did not recognize HOS-A11 cells despite the high frequency of ASV CTL in peripheral blood (Figure S6).

4 | DISCUSSION

We previously reported three PBF-derived CTL epitopes restricted by HLA-B55, HLA-A24 or HLA-A2.[5-7] In the present study, we first identified two PBF-derived HLA-A*11:01-restricted CTL epitopes based on their high binding affinity to HLA-A*11:01 molecules, their ability to bind to TCR and their ability to elicit IFN-γ-producing CTL responses. Importantly, the two epitope-specific CTL can mediate cytotoxic activity against osteosarcoma cells endogenously expressing PBF protein. These results suggest that two epitopes QVT and LSA might be good candidates for use in the design of effective vaccines against osteosarcoma and PBF-related tumors such as Ewing's sarcoma family of tumors,[15] kidney tumors including renal cell carcinoma,[16,17] glioblastoma[18] and breast cancer.[19]

We also characterized two other PBF-derived CTL epitopes, ASV and SVL, that have the ability to induce tetramer-positive T cells. However, such CTL could not produce specific IFN-γ and TNF-α. Because the generation of specific CTL was done with peptides but without additional costimulators and antigen-presenting cells in our experiments, it is possible that CTL induced by these specific peptides are "anergic CTL" or "non-functional CTL" as has been shown for MART-1/HLA A2-specific CTL in both healthy donors and patients.[20,21] A number of studies have shown that the function of

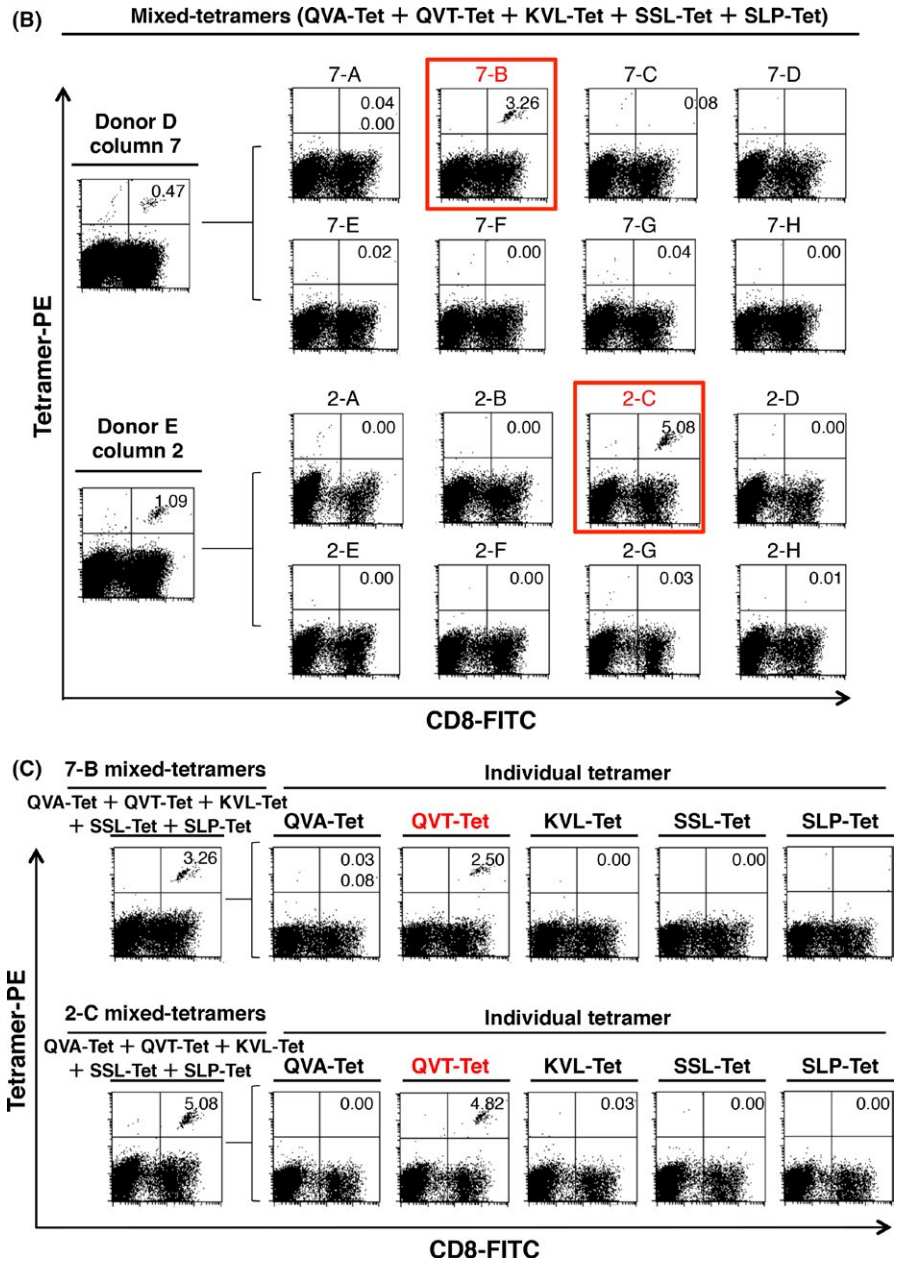


FIGURE 4 continued

in vitro-induced CTL may be influenced by a variety of parameters such as the concentration of peptides, costimulation of antigen-presenting cells, and overexpression of immunosuppressive molecules during in vitro stimulation.[22-27]

Furthermore, we identified a homologous peptide, ASV (R6Q), derived from a microbial ROS family protein. As shown in Figure S1, both ASV and ROS-ASV (R6Q) could induce peptide-specific T-cell responses with similar frequencies, suggesting that ASV and ROS-ASV (R6Q) share the same TCR repertoire. Cross-reactivity of T cells between self-antigens and microbial antigens has previously been described for both $CD4^+$ and $CD8^+$ T cells.[28] Although the ASV peptide mimicking such a pathogen could highly induce peptide-specific CTL, such as like neoantigens, ASV CTL could not lyse HOS-A11 cells. This might be because: (i) ASV CTL fell into anergic or exhausted conditions after MLPC; or (ii) ASV peptide was not naturally presented on osteosarcoma cells in the context of HLA-A11. At least, the high expression of PD1 among ASV-induced CTL after MLPC indicates the possibility of exhaustion (Figure S3).

Web-based in silico prediction has been widely used as a starting peptide collection for T-cell epitope discovery.[29] However, these available databases provide many more potential peptides, making subsequent in vitro screening necessary. Several in vitro screening methods have been developed for selecting true MHC binders, for example, an ELISA-based folding system,[30,31] peptide exchange technology[32,33] and iTopia Epitope Discovery platform.[34,35] Although these ELISA-based methods have been used in T-cell epitope discovery, they suffer from high rates of false-negative and false-positive results. In

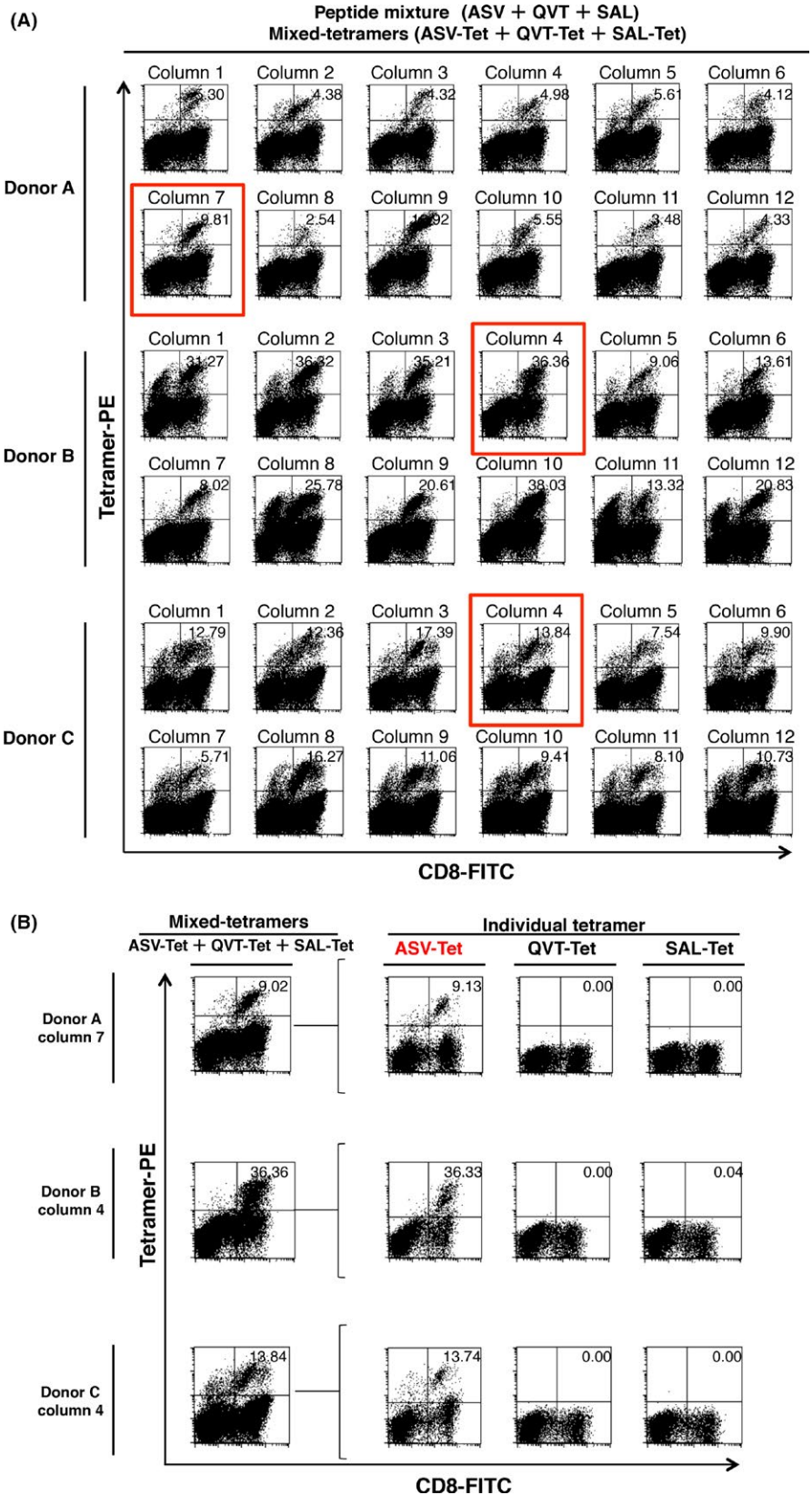


FIGURE 5 MHC-tetramer reactivity with CTL after mixed lymphocyte peptide culture using a mixture of ASV, QVT and SAL peptides. PBMC from three healthy subjects (Donors A, B and C) were stimulated with a mixture of peptides (ASV, QVT and SAL). After peptide mixture stimulation, cells of columns 1-12 were stained with mixed tetramers (ASV-tet, QVT-tet and SAL-tet). Tetramer-positive cells were detected in all columns of Donor A, Donor B and Donor C (A). Column 7 of Donor A, column 4 of Donor B and column 4 of Donor C were selected for an assay with each tetramer, and only ASV-tet-positive cells were detected. B, Frequency of tetramer-positive cells is shown as the percentage of total $CD8^+$ T cells

the present study, we developed an in vitro folding system to directly determine a peptide-MHC complex using HPLC. HPLC is more accurate and reliable for the determination of pMHC levels whereas ELISA shows more variability and less accurate estimation of the levels. Use of the in vitro folding assay might be a useful strategy for T-cell epitope discovery.

In the development of peptide-based immunotherapy strategies, it is becoming increasingly important to cover a large population

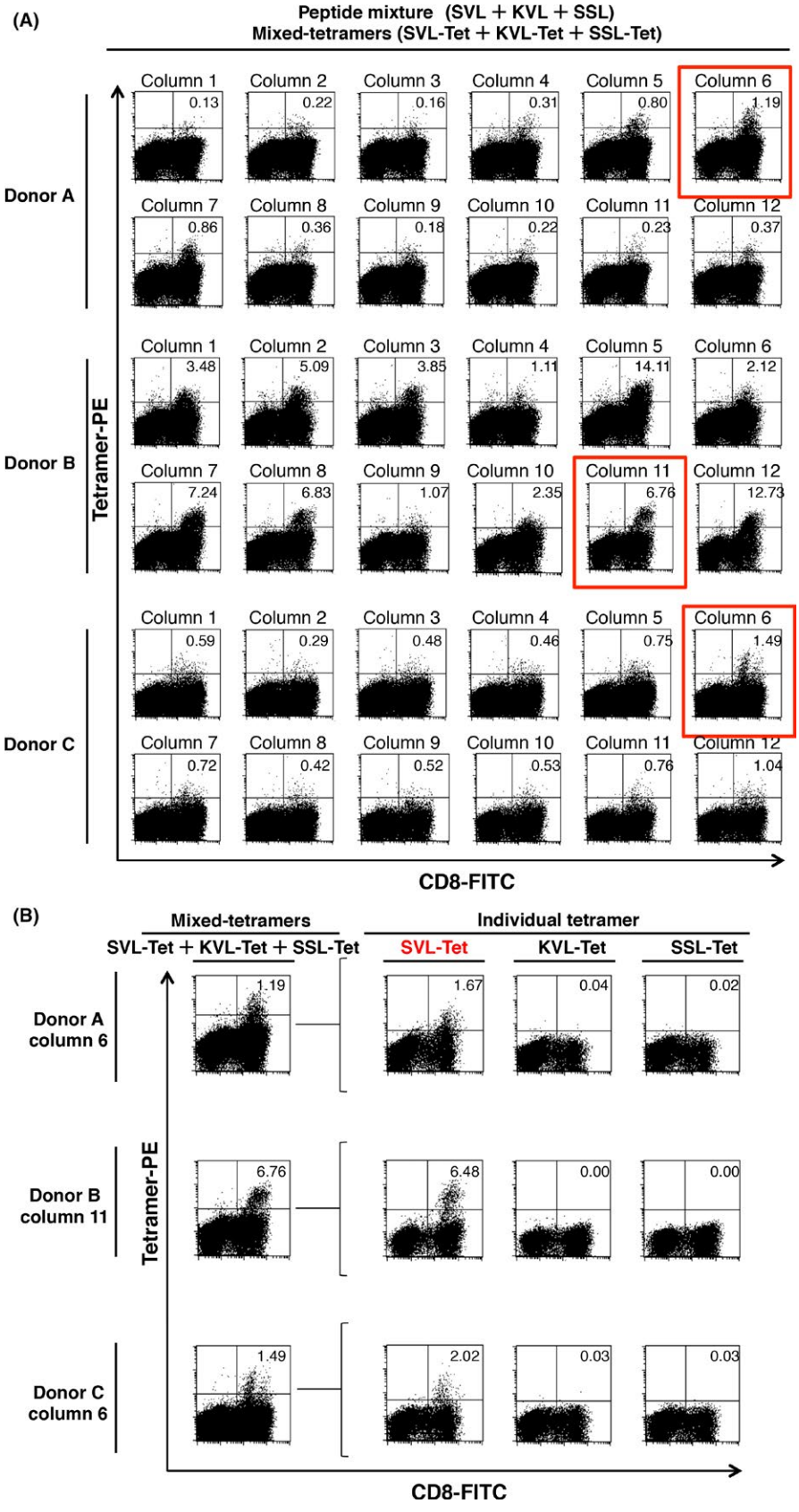


FIGURE 6 MHC-tetramer reactivity with CTL after mixed lymphocyte peptide culture using a mixture of SVL, KVL and SSL peptides. PBMC from three healthy subjects (Donors A, B and C) were stimulated with a mixture of peptides (SVL, KVL and SSL). After peptide mixture stimulation, cells of columns 1-12 were stained with mixed tetramers (SVL-tet, KVL-tet and SSL-tet). Tetramer-positive cells were detected in almost all columns of Donor A, Donor B and Donor C (A). Column 6 of Donor A, column 11 of Donor B and column 6 of Donor C were selected for an assay with each tetramer, and a positive cell population was confirmed only when staining with SVL-tet (B). Frequency of tetramer-positive cells is shown as the percentage of total $CD8^+$ T cells

using several epitopes of multiple alleles. It is well known that the majority of peptide-based cancer vaccines under development are stringently limited to HLA-A2 or HLA-A24-positive patients. To our knowledge, very few HLA-A*11:01-restricted epitopes have been identified until now. We focused on the HLA-A11 allele because of its wide expression in many ethnic populations. Therefore, the newly

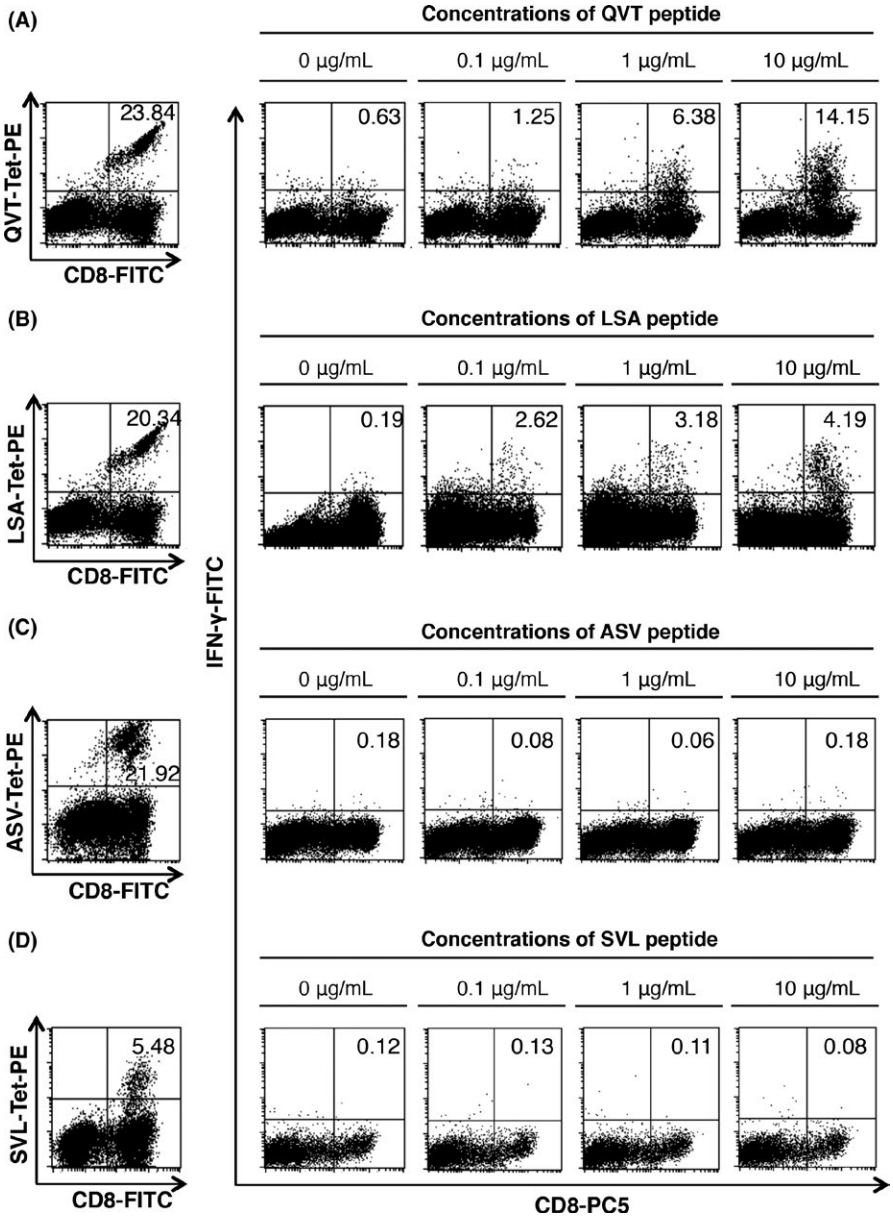


FIGURE 7 Frequency of CD8$^+$interferon (IFN)-γ$^+$ T cells in PBMC after peptide stimulation. CTL were restimulated with a cognate peptide (QVT, LSA, ASV or SVL) at various concentrations in the presence of brefeldin A. QVT and LSA efficiently induced IFN-γ production in a dose-dependent way (A, B). ASV and SVL could not induce specific IFN-γ production (C, D). Percentages of CD8$^+$IFN-γ$^+$ T cells in CD8$^+$ T cells are indicated

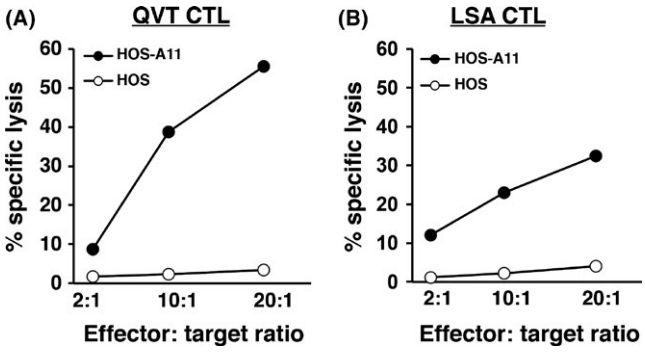


FIGURE 8 Cytotoxicity of QVT- and LSA-induced CTL clones. Cytotoxicity of a QVT-induced CTL clone (QVT CTL) (A) and an LSA-induced CTL clone (LSA CTL) (B) against osteosarcoma HOS (HLA-A11$^-$PBF$^+$) cells and HOS-A11 (HLA-A11$^+$PBF$^+$) cells at the indicated effector : target ratio. HLA, human leukocyte antigen; PBF, papillomavirus binding factor

identified epitopes will benefit broad-spectrum approaches for the development of PBF-targeted immunotherapies.

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CONFLICTS OF INTEREST

We wish to disclose the following conflicts of interest: T. Torigoe received financial support through collaboration with Medical Biological Laboratories Co., Ltd.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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