Detection of novel cancer-testis antigen-specific T-cell responses in TIL, regional lymph nodes, and PBL in patients with esophageal squamous cell carcinoma

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We recently identified three HLA-A2402-restricted epitope peptides derived from cancer-testis antigens (CTA), TTK protein kinase (TTK), lymphocyte antigen 6 complex locus K (LY6K), and insulin-like growth factor (IGF)-II mRNA binding protein 3 (IMP-3) for the development of immunotherapies against esophageal squamous cell carcinoma (ESCC). In order to evaluate their immunotherapeutic potential in ESCC patients, we estimated by ELISPOT assay the TTK-, LY6K-, or IMP-3-specific T-cell immune responses in tumor-infiltrating lymphocytes (TIL), regional lymph node lymphocytes (RLNL), and peripheral blood lymphocytes (PBL) expanded from 20HLA-A2402 (+) ESCC patients, and correlated their immune activity with the expression levels of TTK, LY6K, and IMP-3, and MHC class I in the tumors. Induction of TTK-antigen specific T-cell response in TIL to the peptide-pulsed target cells was detected in 14 out of 20 (70%) cases, while LY6K or IMP-3 specific T-cell activity was observed in 11 of 20 (55%) or in eight of 20 (40%) cases, respectively. Furthermore, T-cell activity in RLNL and PBL was detectable in the similar proportion of the 20 ESCC patients. Interestingly, CTA-specific T-cell immune response was found in 13 of 14 (93%) TIL obtained from ESCC tumors with strong MHC class I expression, while it could be observed only in two of six (33%) TIL from ESCC tumors with weak MHC class I expression. These results strongly suggest the pre-existence of specific T-cell responses to HLA-A24-restricted epitope peptides from TTK, LY6K, and IMP-3 in ESCC patients. Monitoring antigen-specific T-cell responses, as well as the expression levels of MHC class I and epitope CTA in tumors, should be a selection index for application of cancer vaccine therapies to the patients who are likely to show good immune response. (*Cancer Sci* 2008; 99: 1448–1454)

Despite aggressive treatment modalities such as surgical tumor resection with extensive lymphadenectomy and chemo-radiotherapy, the long-term disease control of esophageal squamous cell carcinoma (ESCC) at the advanced stage remains difficult.[1–3] Therefore, immunotherapy such as the utilization of antitumor T cells or antibodies induced by cancer vaccination is extremely appealing.[4] For the development of widely available cancer immunotherapy, it is important to identify a number of immunogenic epitopes that are presented on various types of MHC class I molecules, and that can induce antitumor immune response. Cancer-testis antigens (CTA) such as MAGE, BAGE, GAGE, and NY-ESO-1 are considered to be attractive tumor-associated antigens (TAA) due to their unique expressions in malignant tumors. These antigens are cancer-specific because the cancer-germline genes are silent in normal adult tissues with the exception of male germ cells.[5] Evidence obtained in clinical trials suggest that cancer vaccination with immunogenic epitopes derived from TAA could induce specific T-cell responses in patients with cancers.[6] In addition, tumor regression by immunotherapy was reported in some metastatic renal cell carcinomas, while recurrence of melanoma after surgery was prevented by cancer vaccination with adjuvant setting.[7,8] Although progress in cancer vaccine therapies is significant, its efficacy is still limited to a very small proportion of cancer patients.[6–10] Since the expression of TAA is heterogeneous among patients or individual tumor lesions,[11,12] the development of cancer vaccinations is critically dependent on the identification of ideal TAA that are up-regulated in the majority of cancers, but not in normal tissues, and whose peptide epitopes can induce potent cytotoxic T lymphocyte (CTL) activity.

By using cDNA microarray technology coupled with laser microdissection that can provide comprehensive gene expression profiles of more than 30 000 transcripts between malignant cells and normal counterparts,[13–15] we recently identified three novel HLA-A24-restricted epitope peptides as targets for cancer vaccination against ESCC. These antigenic peptides were derived from three different CTA, TTK protein kinase (TTK), lymphocyte antigen 6 complex locus K (LY6K), and insulin-like growth factor (IGF)-II mRNA binding protein 3 (IMP-3).[16] Our gene expression profile data indicated that *TTK*, *LY6K*, and *IMP-3* were highly expressed in esophageal cancers, while the transcripts of these genes were hardly detectable in normal organs, except the testes and placenta.[17,18] These peptides could stimulate CTL that recognized and killed ESCC cells endogenously expressing these antigens.[16] In order to further develop cancer vaccination with peptides from TTK, LY6K, and IMP-3, the presence of these CTA-specific T-cell responses in cancer patients and their association with the expression of TTK, LY6K, and IMP-3, as well as MHC class I in the tumors, should be examined extensively.

In this study, we evaluated the immunotherapeutic potential of antigenic peptides derived from TTK, LY6K, and IMP-3 by testing the TTK-, LY6K-, or IMP-3-specific T-cell responses in tumor-infiltrating lymphocytes (TIL), regional lymph node lymphocytes (RLNL), and PBL obtained from HLA-A2402 (+) patients with ESCC. We also examined the correlation of CTA-specific T-cell activity with the expression levels of TTK, LY6K, IMP-3, and MHC class I in the tumors.

Materials and Methods

Patients and samples. Twenty HLA-A2402 (+) patients with histologically diagnosed primary ESCC who were treated at the First Department of Surgery, University of Yamanashi Hospital, were enrolled in this study with written informed consent. None of the patients had received any treatment before surgery

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(preoperative radiotherapy, chemotherapy, or immunotherapy) and all patients had undergone esophagectomy with two-field ($n = 7$) or three-field ($n = 13$) lymph node dissection. This study and the use of all clinical materials were approved by individual institutional ethical committees.

Immunohistochemical analysis. Four-μm thick sections of archival, formalin-fixed, paraffin-embedded tissue block (ESCC and adjacent normal esophagus) were used for immunohistochemical analysis. To investigate the expression of TTK, LY6K, and IMP-3 protein in clinical ESCCs, we stained tissue sections using ENVISION + Kit/HRP (DakoCytomation, Glostrup, Denmark) as previously described.[18] A rabbit polyclonal antihuman TTK antibody (NB 100-463, 70 mg/mL; Nobus Biologicals, Littleton, CO, USA), a rabbit polyclonal antihuman LY6K antibody (TM38, originally generated to recombinant LY6K;[18] 4 mg/mL), or a goat polyclonal antihuman IMP-3 antibody (sc47892, 2.5 mg/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to deparaffinized sections after blocking of endogenous peroxidase. Then, the sections were incubated with HRP-labeled antirabbit or antigoat IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin. For HLA-class I staining, the sections were dewaxed, followed by antigen retrieval with Epitope Retrieval Solution (10 mmol citrate buffer (pH 6.0) (DakoCytomation) in an autoclave (121°C, 20 min). Endogenous peroxidase was blocked by Chemmate Peroxidase Blocking Solution (DakoCytomation). The primary antibody EMR8-5 (anti-HLA class I heavy chain, diluted by phosphate-buffered saline, 1:100; Cosmo Bio Co., Tokyo, Japan) was applied to the sections at 4°C overnight. Thereafter, the sections were incubated with streptavidin-biotin complex (Simple Stain MAX-PO kit; Nichirei, Tokyo, Japan) for 30 min. The sections were then treated with 3,3′-diaminobenzidine (DakoCytomation) for 5 min, and counterstained with hematoxylin. Negative control staining was performed with isotype control monoclonal antibodies (mAbs) (DakoCytomation). Three independent investigators assessed the staining positivity semiquantitatively without prior knowledge of clinicopathological or immunological data. The intensity of TTK, LY6K, IMP-3, or HLA class I staining was evaluated using following criteria: strong positive (strong), dark brown staining in more than 50% of tumor cells completely obscuring cytoplasm; weak positive (weak), any lesser degree of brown staining appreciable in tumor cells; absent, no appreciable staining in tumor cells.

Peptides. Epitope peptides derived from TTK (*TTK*-567, SYRNEIAYL), LY6K (*LY6K*-177, RYCNLEGPPI), and IMP-3 (*IMP-3*-508, KTVNELQNL) that could bind to HLA-A2402 molecule were synthesized as described elsewhere.[16] The purity (90%) and identity of the peptides were determined by analytical high-performance liquid chromatography and mass spectrometric analysis. Peptides were dissolved in dimethylsulfoxide at the concentration of 20 mg/mL and stored at –80°C. HIV peptide with HLA-A2402 binding capacity (ILKEPVHGV) was used as a negative control peptide.

Cell lines. The human B-lymphoblastoid cell line TISI cells expressing HLA-A24 on their surface were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 5% heat inactivated fetal calf serum (FCS) (Invitrogen, Grand Island, NY, USA) and 1% penicillin and streptomycin. COS-7 cells were cotransfected with the vectors expressing *TTK*, *LY6K*, *IMP-3*, *HLA-A2402*, or *HLA-A0201* using FuGENE6 (Roche, Indianapolis, IN, USA) reagent. Thereafter, 3×10^5 cells/well were incubated in Dulbecco's modified Eagle medium (Sigma-Aldrich) with 10% FCS and 1% antimycobiotics (Sigma-Aldrich) in six-well flat bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Two days after culture, the cells were used as target cells in a gamma-interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay.

Lymphocytes preparation. PBL were isolated with Ficoll-Paque Plus (GE Healthcare Bio-sciences, Piscataway, NJ, USA) density gradient solution. Tumor tissue and regional lymph nodes were obtained from ESCC patients during surgery and homogenized by mechanical mincing with X-VIVO15 medium (Cambrex, East Rutherford, NJ, USA). Then, cell mixtures were passed through a cell strainer (Becton Dickinson Labware) and diluted as a single-cell suspension with X-VIVO15. Subsequently, the single-cell suspension was purified by centrifugation with Ficoll-Paque Plus.

***In vitro* T-cell culture and ELISPOT assay.** TIL, RLNL, and PBL (5×10^5 cells/well) obtained from ESCC patients were cultured in X-VIVO15 medium with 100 U/mL rIL-2 (Peprotech, Rocky Hill, NJ, USA) in 48-well flat bottom plates (Becton Dickinson Labware) for 2 days without any peptide pulse. On days 2 and 9, the cultured cells were added with TTK-, LY6K-, or IMP-3-peptides (10 μg/mL), or not treated with peptide (negative control). On day 15, the cultured cells were subjected to IFN-γ ELISPOT assay as responder cells. To select and culture TIL, plastic adherent cells including tumor cells and stroma cells were partially removed from the cultured cells that were fed with medium in the presence of interleukin (IL)-2. Eighty-five to 95% of the cultured cells on day 15 derived from TIL were confirmed to be CD3-positive as defined by FACS staining.

For detecting antigen-specific immune response, ELISPOT assay was performed with the human IFN-γ ELISPOT kit (Mabtech, Nacka Strand, Sweden). Ninety-six-well plates with nitrocellulose membranes (Millipore, Molshelm, France) were precoated with primary anti-IFN-γ antibody (1-D1K) at 4°C overnight. The plates were then prereacted with X-VIVO15 containing 1% human serum albumin (Sigma-Aldrich). TISI cells (2×10^4/well) were incubated for 24 h in triplicate with responder cells (2×10^3/well), and CTA peptides (2, 20, or 50 μg/mL) or HIV-peptide (20 μg/mL) in a total of 200 μL/well of X-VIVO15. Further, COS-7 cells (2×10^3/well) transfected with individual genes (see above) and responder cells (2×10^3/well) were mixed and incubated in a final volume of 200 μL/well of X-VIVO15 for 24 h in triplicate. These cell mixtures were treated with biotinylated secondary anti-IFN-γ antibody (7-B6-1) and incubated for 2 h. Then the plates were incubated with streptavidin-alkaline phosphatase reagent and stained with NBT and BCIP (Sigma-Aldrich). The spots were quantified with the auto-analyzing system, KS ELISPOT Compact (Zeiss, Göttingen, Germany). Positivity of peptide-specific T-cell response was defined as follows: the number of spots in a specific peptide (TTK, LY6K, or IMP-3 at the final concentration of 20 μg/mL)–pulsed TISI was ≥ twice as many as that in HIV peptide-pulsed TISI. We defined the assessable lower limit of the spot per well as ≥40 spots.

Statistical analysis. Differences between values were determined using the non-paired Student's *t*-test. Significance was considered at $P < 0.05$.

Results

TTK, LY6K, IMP-3, and MHC class I protein expression in ESCC. We semiquantitatively evaluated by immunohistochemical staining the expression levels of TTK, LY6K, IMP-3, and MHC class I protein in surgically resected ESCC tissues from 20HLA-A2402(+) patients enrolled in this study. Representative immunohistochemical staining is shown in Figure 1, and the characteristics of 20 patients (age, gender, and pStage) are described in Table 1. We confirmed that the expression of TTK, LY6K, and IMP-3 was frequently observed in ESCC tissues (100% for TTK, 90% for LY6K, and 90% for IMP-3; Table 1), while no staining was observed in adjacent esophageal tissues (Fig. 1). Simultaneous expression of two or all of these three CTA was seen in 19 out of 20 patients. In addition, MHC class

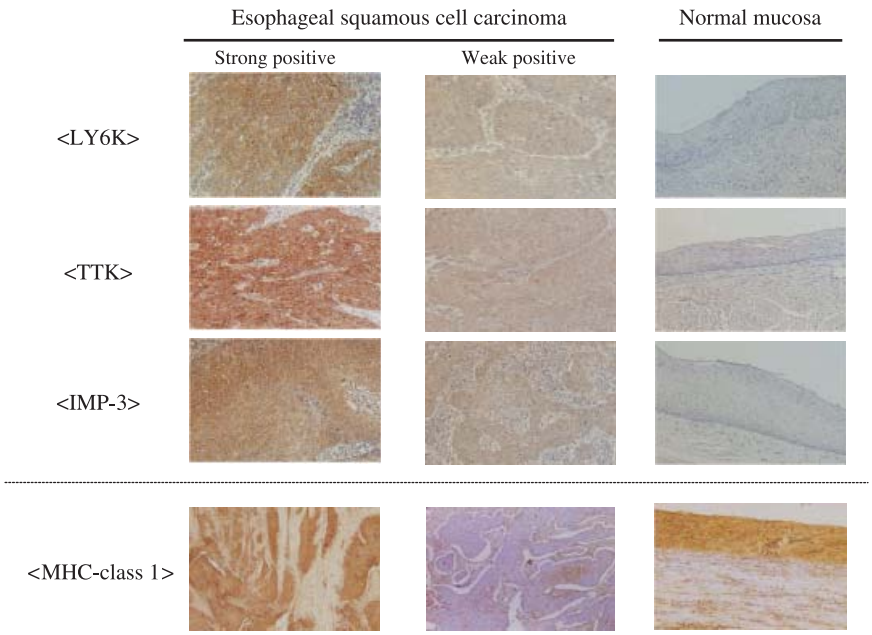

Fig. 1. Representative immunohistochemical staining of esophageal squamous cell carcinoma using antibodies to LY6K, TTK, IMP-3, and MHC class I.

Table 1. Peptide-specific T-cell responses in relation to TTK, LY6K, IMP-3, and MHC class expression

Patient	Age (year)/Gender (M/F)	Stage†	Peptide-specific T-cell responses									Immunohistochemical staining			
			TIL			RLNL			PBL			TTK	LY6K	IMP-3	MHC class
			TTK	LY6K	IMP-3	TTK	LY6K	IMP-3	TTK	LY6K	IMP-3				
1	68/M	IIA	+	+	+	+	+	+	+	–	–	Strong	Weak	Absent	Strong
2	76/M	IIB	–	–	–	–	–	–	–	–	–	Strong	Strong	Strong	Weak
3	73/M	IV	+	+	+	+	+	+	+	+	+	Strong	Strong	Strong	Strong
4	54/M	III	+	+	+	+	+	+	+	+	+	Strong	Strong	Strong	Strong
5	61/M	III	+	–	–	+	–	–	+	–	–	Strong	Absent	Absent	Weak
6	62/M	III	+	–	–	+	–	–	+	–	–	Strong	Weak	Weak	Strong
7	57/M	I	+	+	–	+	+	–	+	+	–	Strong	Strong	Strong	Strong
8	63/M	I	–	–	–	–	–	–	–	–	–	Strong	Weak	Weak	Weak
9	66/M	IIB	+	+	+	+	+	+	+	+	+	Strong	Strong	Strong	Strong
10	79/M	I	+	+	–	+	+	–	+	+	–	Strong	Weak	Strong	Strong
11	59/M	IIB	–	–	–	–	–	–	–	–	–	Strong	Strong	Weak	Weak
12	62/M	IIA	+	–	+	+	–	+	+	–	+	Weak	Absent	Weak	Strong
13	69/M	0	+	–	+	–	–	+	–	–	+	Strong	Weak	Weak	Strong
14	75/M	I	–	–	–	–	–	–	–	–	–	Strong	Strong	Weak	Weak
15	62/M	III	+	+	–	+	+	–	+	+	–	Strong	Strong	Weak	Weak
16	74/M	IIA	–	+	–	–	+	–	–	+	–	Strong	Weak	Weak	Strong
17	72/M	0	–	–	–	–	–	–	–	–	–	Strong	Weak	Strong	Strong
18	60/M	IIB	+	+	+	+	+	+	+	+	+	Strong	Weak	Strong	Strong
19	71/M	IIA	+	+	+	+	+	+	+	+	+	Strong	Weak	Weak	Strong
20	63/M	I	+	+	–	+	+	–	+	+	–	Strong	Weak	Strong	Strong
Summary of T-cell response rate			14/20	11/20	8/20	13/20	11/20	8/20	13/20	10/20	7/20				

†Stage according to the TNM classification for esophageal cancer (UICC).
Absent, absent expression; PBL, peripheral blood lymphocytes; RLNL, regional lymph-node lymphocytes; strong, strong expression; TIL, tumor-infiltrating lymphocytes; weak, weak expression.

I, an elemental molecule for antigen presentation, was highly expressed in 14 out of 20 cases, while it was down-regulated in six out of 20 cases (Table 1).

Peptide-specific T-cell response in TIL, RLNL, and PBL. Lymphocytes derived from TIL, RLNL, and PBL were incubated with rIL-2 for 14 days and stimulated with antigenic peptides (*TTK*-567, *LY6K*-177, or *IMP-3*-508) two times in a week interval (days 2 and 9) prior to ELISPOT assay. On day 15, the cultured cells were subjected to IFN-γ ELISPOT assay. As demonstrated in Figure 2a (representative data of TIL from an ESCC patient, case 6), potent and specific IFN-γ production by cultured lymphocytes against peptide-pulsed target cells was detected; the cultured TIL stimulated with *TTK*-567 showed significant INF-γ production against the TTK-peptide pulsed targets (TISI pulsed with TTK peptides), while the cultured cells stimulated with *LY6K*-177 or *IMP-3*-508 did not confer immune activity against corresponding peptide-pulsed targets (TISI pulsed with LY6K or IMP-3), suggesting the pre-existence of specific T-cell responses to HLA-A2402-restricted TTK-epitope peptides, but not to LY6K and IMP-3 in this patient. In addition, as indicated by ELISPOT

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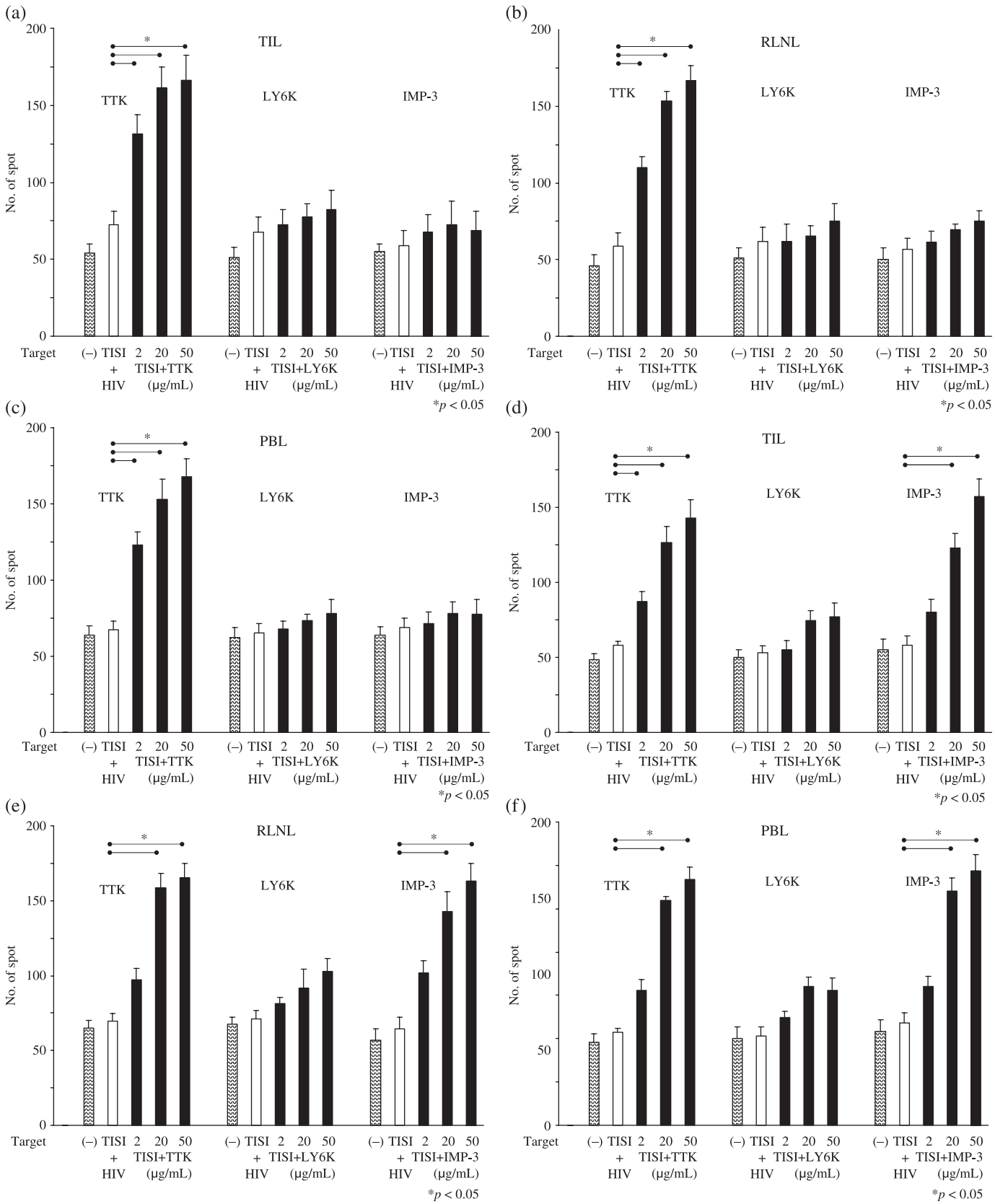

Fig. 2. Peptide-specific T-cell responses in cultured T cells from TIL, RLNL, and PBL. Lymphocytes in TIL, RLNL, and PBL from patients (a–c, case 6; d–f, case 12) were incubated with interleukin (IL)-2 for 14 days and stimulated with epitope peptides (*TTK*-567, *LY6K*-177, or *IMP-3*-508) two times per week (days 2 and 9) prior to enzyme-linked immunosorbent (ELISPOT) assay. On the 15th day, the cultured cells were subjected to ELISPOT assay. TISI cells (2×10^4/well) were incubated for 24 h in triplicate with responder cells (2×10^3/well), and individual CTA peptides (2, 20, or 50 μg/mL) or HIV peptides (20 μg/mL).

Table 2. Peptide-specific T-cell responses by enzyme-linked immunosorbent (ELISPOT) assay

Peptide stimulation (*in vitro*)	TIL						RLNL						PBL					
	TTK		LY6K		IMP-3		TTK		LY6K		IMP-3		TTK		LY6K		IMP-3	
Antigen on target	+ HIV	+ TTK	+ HIV	+ LY6K	+ HIV	+ IMP-3	+ HIV	+ TTK	+ HIV	+ LY6K	+ HIV	+ IMP-3	+ HIV	+ TTK	+ HIV	+ LY6K	+ HIV	+ IMP-3
Patient																		
1	57.3 ± 8.1	132.0 ± 7.2	55.7 ± 4.2	128.3 ± 10.0	53.3 ± 5.5	115.3 ± 7.2	66.3 ± 12.5	148.7 ± 18.0	69.0 ± 5.6	142.3 ± 14.6	65.7 ± 3.8	134.3 ± 11.0	59.7 ± 11.7	149.7 ± 13.9	64.7 ± 6.0	115.3 ± 12.9	62.7 ± 7.6	99.3 ± 7.8
2	73.3 ± 7.1	114.7 ± 11.0	76.3 ± 4.5	103.7 ± 7.8	66.7 ± 5.0	116.7 ± 10.1	78.7 ± 9.1	125.7 ± 12.7	75.7 ± 3.5	110.7 ± 7.8	75.7 ± 9.5	115.0 ± 5.3	66.3 ± 8.1	91.7 ± 3.1	61.7 ± 6.0	100.7 ± 4.5	64.3 ± 8.5	114.3 ± 8.1
3	58.7 ± 2.1	129.3 ± 9.5	62.7 ± 7.6	132.7 ± 6.7	60.7 ± 4.5	126.7 ± 8.7	60.3 ± 7.0	130.3 ± 3.1	59.7 ± 5.1	127.7 ± 7.6	64.7 ± 7.0	138.7 ± 9.1	54.3 ± 4.0	129.0 ± 10.5	58.3 ± 5.5	136.3 ± 10.4	51.7 ± 4.5	115.0 ± 14.5
4	55.3 ± 6.7	132.3 ± 6.1	57.7 ± 3.2	137.7 ± 8.5	62.7 ± 8.3	135.0 ± 11.5	62.3 ± 4.5	144.3 ± 8.7	58.7 ± 3.5	141.3 ± 4.9	63.3 ± 5.1	135.7 ± 8.7	53.7 ± 2.5	119.7 ± 10.4	54.3 ± 6.8	115.0 ± 5.3	52.7 ± 6.0	110.7 ± 5.5
5	55.7 ± 5.0	124.7 ± 6.0	59.7 ± 7.0	77.0 ± 11.1	52.7 ± 4.5	65.3 ± 9.3	61.7 ± 4.0	138.7 ± 10.0	58.7 ± 6.7	78.3 ± 8.1	55.3 ± 2.1	73.7 ± 8.1	54.0 ± 4.6	118.3 ± 10.0	50.3 ± 5.8	59.7 ± 5.7	52.7 ± 5.1	62.3 ± 10.3
6	71.3 ± 8.6	160.3 ± 14.3	65.3 ± 9.5	75.3 ± 8.1	58.3 ± 9.7	72.3 ± 15.4	59.0 ± 8.7	153.3 ± 6.1	62.0 ± 8.9	65.3 ± 7.0	57.3 ± 6.8	69.7 ± 3.2	67.3 ± 5.5	153.3 ± 13.1	64.7 ± 6.0	73.7 ± 3.8	68.7 ± 6.4	78.3 ± 7.5
7	61.7 ± 8.1	135.7 ± 13.7	63.3 ± 5.0	139.3 ± 12.0	64.7 ± 5.0	121.3 ± 9.3	56.0 ± 2.6	135.7 ± 6.7	59.7 ± 5.5	129.7 ± 15.9	61.7 ± 5.7	106.7 ± 7.5	64.3 ± 3.5	137.0 ± 9.8	58.0 ± 6.2	135.7 ± 6.1	65.3 ± 3.8	122.7 ± 7.5
8	55.3 ± 7.0	89.3 ± 11.7	57.7 ± 5.7	84.3 ± 11.9	62.3 ± 5.1	80.3 ± 8.0	61.7 ± 5.5	80.3 ± 12.4	57.3 ± 5.1	76.3 ± 4.5	52.3 ± 5.0	78.3 ± 6.5	63.3 ± 6.4	87.7 ± 11.0	57.0 ± 4.0	74.7 ± 2.5	59.3 ± 6.5	78.7 ± 5.9
9	53.3 ± 8.6	136.7 ± 7.5	55.7 ± 4.6	126.7 ± 10.2	52.7 ± 4.5	118.7 ± 7.6	67.3 ± 2.6	145.3 ± 10.0	65.3 ± 6.0	148.7 ± 8.7	56.0 ± 6.0	139.7 ± 6.1	59.0 ± 7.2	146.3 ± 7.6	57.3 ± 4.5	129.7 ± 6.4	62.0 ± 6.1	138.3 ± 7.8
10	57.3 ± 6.9	136.3 ± 7.9	53.7 ± 3.1	117.7 ± 9.5	61.3 ± 5.7	106.7 ± 8.5	75.3 ± 8.6	166.3 ± 12.2	72.7 ± 5.7	152.3 ± 9.3	77.3 ± 4.7	143.7 ± 5.7	51.3 ± 1.7	112.7 ± 3.7	50.3 ± 4.0	105.3 ± 3.8	54.7 ± 4.0	99.7 ± 16.6
11	62.3 ± 4.2	82.3 ± 5.5	63.7 ± 3.8	74.3 ± 2.1	60.7 ± 5.5	77.0 ± 5.6	70.3 ± 8.3	87.0 ± 9.5	67.3 ± 6.0	77.0 ± 2.6	55.3 ± 3.2	81.7 ± 9.1	56.7 ± 3.5	81.0 ± 6.0	53.0 ± 4.6	73.7 ± 3.2	54.3 ± 5.7	64.7 ± 5.5
12	58.3 ± 2.5	126.3 ± 11.1	53.3 ± 4.6	74.3 ± 7.0	58.0 ± 6.2	123.0 ± 9.6	69.7 ± 5.1	158.7 ± 9.8	71.3 ± 5.5	91.7 ± 12.7	64.3 ± 7.6	142.7 ± 13.5	53.5 ± 2.5	129.7 ± 2.5	51.3 ± 5.7	80.3 ± 5.1	59.3 ± 5.8	135.3 ± 7.5
13	73.3 ± 2.3	164.3 ± 6.1	68.3 ± 6.5	118.0 ± 10.5	71.7 ± 6.5	148.7 ± 5.3	67.7 ± 8.2	130.7 ± 10.0	64.7 ± 9.1	114.7 ± 11.0	63.7 ± 8.1	136.3 ± 6.5	59.0 ± 2.6	116.7 ± 17.4	57.7 ± 4.7	88.7 ± 6.7	53.7 ± 7.5	123.7 ± 4.1
14	59.7 ± 3.2	67.3 ± 7.8	58.7 ± 7.1	70.0 ± 8.5	55.3 ± 4.5	67.3 ± 1.7	71.0 ± 9.2	86.3 ± 1.2	66.7 ± 7.1	75.0 ± 7.0	65.0 ± 5.2	77.0 ± 7.0	60.7 ± 3.2	87.7 ± 6.1	57.3 ± 3.5	74.7 ± 5.5	61.7 ± 6.5	77.7 ± 3.1
15	57.3 ± 5.8	118.3 ± 9.5	56.0 ± 5.6	116.3 ± 13.9	59.3 ± 6.1	95.7 ± 6.1	72.3 ± 5.1	150.7 ± 11.8	69.3 ± 5.9	147.0 ± 9.9	70.3 ± 7.1	119.3 ± 13.7	52.7 ± 7.2	123.0 ± 7.1	51.7 ± 6.4	116.7 ± 10.1	56.3 ± 5.5	86.0 ± 5.7
16	71.0 ± 6.0	136.7 ± 7.1	69.0 ± 5.3	146.0 ± 7.9	67.3 ± 8.5	85.7 ± 10.8	64.7 ± 4.7	123.3 ± 10.0	63.3 ± 5.1	135.3 ± 10.5	58.7 ± 8.5	86.7 ± 11.0	51.7 ± 3.7	94.0 ± 7.0	53.3 ± 4.5	114.3 ± 8.1	58.0 ± 5.0	67.3 ± 8.5
17	54.7 ± 4.7	91.3 ± 9.0	58.7 ± 5.0	85.3 ± 3.8	65.7 ± 6.1	92.0 ± 7.8	72.7 ± 4.8	137.0 ± 9.8	77.3 ± 10.0	125.3 ± 9.1	76.3 ± 8.5	132.7 ± 8.1	53.7 ± 4.0	96.3 ± 6.4	56.7 ± 8.1	73.0 ± 7.5	62.7 ± 4.0	87.3 ± 10.6
18	63.0 ± 4.0	146.0 ± 7.0	59.3 ± 6.5	141.3 ± 4.9	61.0 ± 5.6	137.7 ± 10.0	74.7 ± 5.1	166.7 ± 8.1	74.0 ± 4.4	155.0 ± 11.1	69.7 ± 6.1	157.3 ± 11.7	60.3 ± 8.1	137.7 ± 9.6	58.3 ± 7.1	124.7 ± 8.5	61.7 ± 5.9	139.0 ± 7.8
19	66.3 ± 9.5	144.0 ± 9.2	65.7 ± 3.1	134.7 ± 14.3	65.3 ± 7.5	135.0 ± 6.2	71.3 ± 6.8	164.0 ± 5.3	69.0 ± 4.0	145.3 ± 9.1	72.7 ± 4.5	148.7 ± 11.7	57.3 ± 7.5	134.3 ± 4.7	58.0 ± 7.5	122.7 ± 6.7	62.7 ± 5.0	129.7 ± 4.5
20	51.3 ± 4.0	119.3 ± 8.1	52.7 ± 6.5	107.3 ± 7.5	55.7 ± 3.1	96.3 ± 7.5	72.0 ± 5.6	157.0 ± 9.2	69.7 ± 6.1	146.7 ± 6.0	65.3 ± 7.0	124.0 ± 11.5	61.3 ± 2.5	138.7 ± 9.5	60.7 ± 6.1	125.7 ± 12.7	64.3 ± 7.6	107.7 ± 7.5

The number of spots on ELISPOT assay were demonstrated as mean ± SD for TISI targets pulsed with peptides (20 μg/mL).
PBL, peripheral blood lymphocytes; RLNL, regional lymph-node lymphocytes; TIL, tumor-infiltrating lymphocytes.

assay using RLNL and PBL from the same patient, specific T-cell responses to TTK-peptide were seen in not only TIL, but also in RLNL and PBL (Fig. 2b,c). On the other hand, specific T-cell responses to TTK- and IMP-3-peptides were seen in TIL, RLNL, and PBL from another patient (case 12; Fig. 2d–f).

As expected, TIL, RLNL, and PBL from ESCC patients ($n = 20$) precultured with rIL-2 for 14 days without any peptide stimulation did not show any immunologic response against TISI pulsed with any CTA peptides (data not shown). In addition, PBL from healthy volunteers ($n = 10$) stimulated with the antigenic peptides did not produce specific INF-γ against the TISI pulsed with any CTA peptides (data not shown). These results indicate the suitability of our *in vitro* assay in the detection of patient-specific and CTA-specific T-cell responses.

The ELISPOT data in TIL, RLNL, and PBL from ESCC patients ($n = 20$) are shown in Table 2, and the specific T-cell responses in relation to CTAs and MHC class I expression are summarized in Table 1. Induction of TTK-antigen specific T-cell response in TIL was detected in 14 out of 20 (70%) cases, while LY6K or IMP-3 specific T-cell activity was observed in 11 of 20 (55%) or in eight of 20 (40%) cases, respectively. Furthermore, the peptide-specific T-cell responses were observed in almost the same incidence of RLNL and PBL (Tables 1 and 2). These results imply that TTK-, LY6K-, and IMP-3-specific T-cell responses were frequently induced in both loco-regional and systemic immunity of ESCC patients.

We further analyzed by IFN-γ ELISPOT assay antigen-specific T-cell activity against COS-7 cells transfected with plasmids expressing each CTA and/or those expressing HLA-A2402 or HLA-A0201. We confirmed that the cultured cells in TIL from patient 6 showed specific and significant IFN-γ release against COS-7 cells cotransfected with vectors for HLA-A24 expression and those for TTK, while they did not immunologically respond to LY6K-, IMP-3-, or other control gene transfectants (Fig. 3a). The same result was also seen in cultured cells from RLNL and PBL (Fig. 3b,c). These data indicate that cultured cells derived from TIL, RLNL, and PBL could recognize TTK antigens which are naturally processed and present on the cell surface in context with HLA-A2402.

Correlation between peptide-specific T-cell response and MHC class I expression. As shown in Table 1, there was no perfect correlation between the peptide-specific T-cell responses and the CTA expression in the tumors. For example, even if the tumor strongly expressed the CTA, antigen-specific T-cell responses to the CTA were not always observed in the T cells expanded from the patients, and vice versa. Therefore, we compared the T-cell response with the levels of MHC class I expression. Interestingly, TIL obtained from ESCC tumors with strong MHC class I expression ($n = 14$) were likely to respond to the CTA peptides (13 out of 14 [93%] cases), while CTA-specific T-cell activity was found only in two of six (33%) TIL from ESCC tumors with weak MHC class I expression (Table 3). These results indicate that high levels of MHC class I expression on the tumor might be one of the essential factors for the *in vivo* induction of TTK-, LY6K-, or IMP-3-specific T-cell responses.

Table 3. Correlation between peptide-specific T-cell response in TIL and HLA-class expression

Immune response TIL	HLA-class expression	
	Strong ($n = 14$)	Weak ($n = 6$)
Positive ($n = 15$)	13	2
Negative ($n = 5$)	1	4

$P < 0.02$ by Fisher's exact probability test.
Strong, strong expression; TIL, tumor-infiltrating lymphocytes; weak, weak expression.

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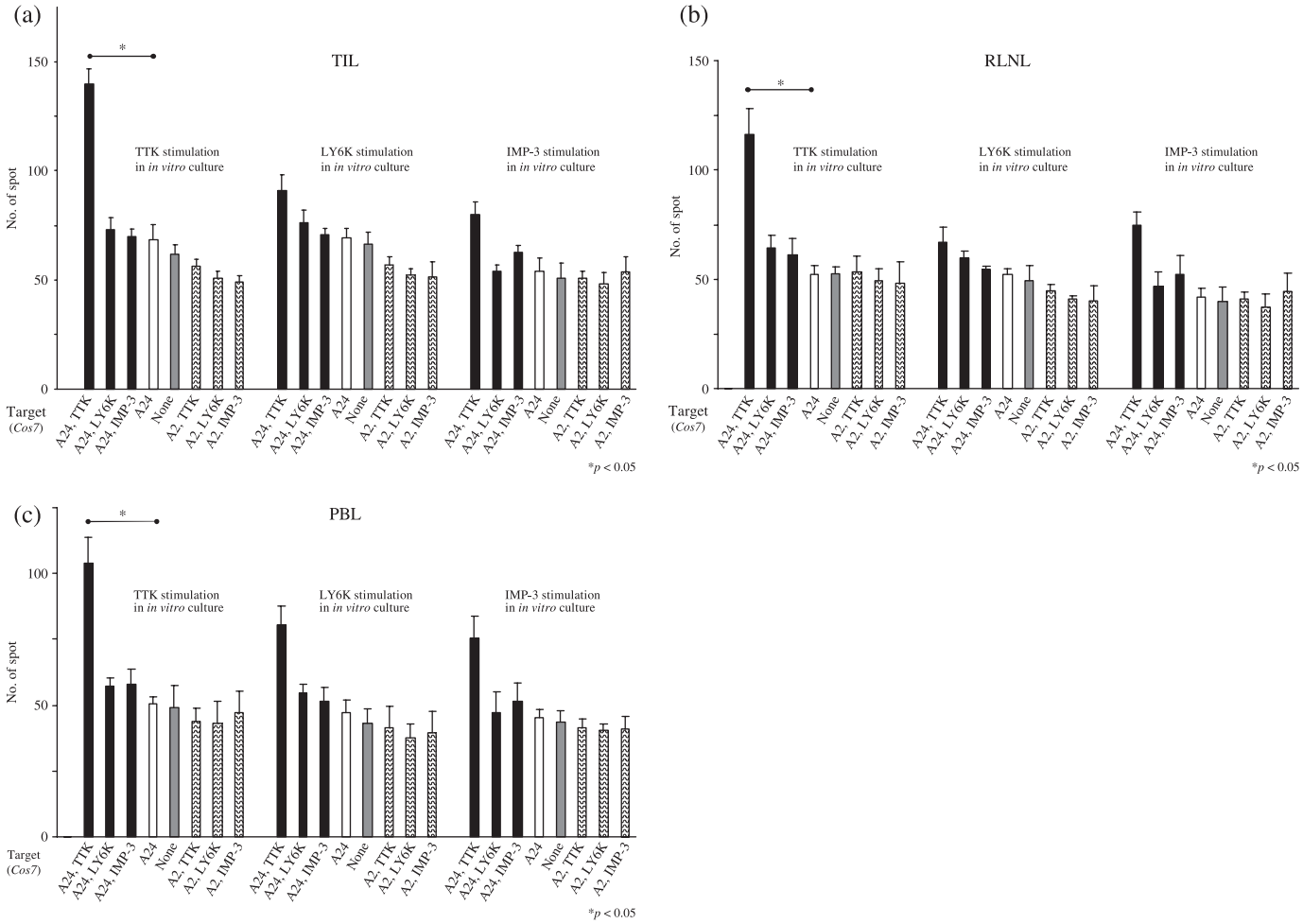

Fig. 3. Specific immune response of cultured T cells from TIL, RLNL, and PBL to COS-7 cells transfected with *HLA-A2402* and *TTK*, *LY6K*, and *IMP-3* genes. Lymphocytes in TIL, RLNL, and PBL obtained from one patient (a–c, case 6) were incubated with interleukin (IL)-2 for 14 days and stimulated with cognate peptides two times per week (days 2 and 9) prior to enzyme-linked immunosorbent (ELISPOT) assay. On day 15, cultured cells were subjected to ELISPOT assay. COS-7 cells (2×10^3/well) transfected with the combination of *TTK, LY6K, IMP-3, HLA-A2402(A24)* or *HLA-A0201(A2)* vectors, and responder cells (2×10^3/well) were mixed and incubated for 24 h in triplicate.

Discussion

In the present study, we described specific T-cell responses to epitope peptides derived from TTK, LY6K, and IMP-3, which were frequently observed in TIL, RLNL, and PBL from patients with HLA-A2402(+) ESCC. In addition, we evaluated the correlation between antigen-specific T-cell responses, and the expression levels of both CTA and MHC class I expression in tumors.

We have been accumulating comprehensive gene expression profiles of more than 1000 human cancers by use of the cDNA microarray covering more than 30 000 human transcripts.[13–15,17] Using this database with subsequent immunological assays, we successfully identified three novel HLA-A2402-restricted epitope peptides derived from TTK, LY6K, and IMP-3.[16] *TTK*, *LY6K*, and *IMP-3* were highly expressed in the great majority of ESCC, but scarcely expressed in normal tissues.[17,18] We reported that higher expression of LY6K was associated with poor prognosis for patients with ESCC, and that LY6K was indispensable for cell growth and/or survival of cancer cells.[18] On the other hand, it was suggested that TTK could be associated with cell proliferation and the cell cycle,[19] and IMP-3 might be involved in cell adhesion and tumor invasion.[20] In this study, we confirmed simultaneous over-expression of TTK, LY6K, and/or IMP-3 proteins in 19 out of 20 patients. Since the evidence clearly suggests that TTK, LY6K, and IMP-3 could play fundamental roles in the process of cancer cell growth/survival, these CTA might be good therapeutic target molecules for cancer immunotherapy.

When the antigenic epitope from CTA is applied to cancer vaccination, it is important to prove the presence of precursor T cells specific for the tumor antigens in cancer patients. Evidence of pre-existing CTA-specific immune response supports the idea that cancer vaccination is able to boost strong immune responses specific for CTA and may subsequently exert a therapeutic effect. In this study, we confirmed that TTK-, LY6K-, and IMP-3-specific T-cell responses were frequently induced in TIL, RLNL, and PBL from HLA-A2402(+) patients with ESCC, suggesting that these CTA-specific T-cell immunities were not tolerated in ESCC patients. The power in detecting antigen-specific T-cell response is known to be dependent on how T cells were stimulated with *in vitro* culture and then applied to the assay systems.[21–23] We could successfully detect the peptide-specific T-cell response by two-round re-stimulations with cognate peptide alone *in vitro*. As is well known, it was quite difficult to detect specific T-cell response by using conventional TAA such as MAGE, HER2, or CEA under such conditions as ours.[10,24,25] These results suggested that TTK-, LY6K-, and IMP-3-specific

T-cell responses were pre-existed in ESCC patients with relatively high incidence. Furthermore, TTK-, LY6K-, and IMP-3-specific T-cell responses were also observed in RLNL and PBL as well as in TIL. TTK-, LY6K-, and IMP-3-specific T-cell responses might be induced not only loco-regionally, but also systemically in ESCC patients.

In the present study, there was no perfect correlation between the peptide-specific T-cell responses and the CTA expression in tumors. One can speculate that this may be due to the limitation of CTA detection by immunohistochemistry, or insufficient sensitivity of the ELISPOT assay for detecting antigen specific response. However, we found a significant correlation between the positivity of peptide-specific T-cell responses to TTK, LY6K, or IMP-3, and strong MHC class I expression on ESCC. Thus, it is likely that precursor T cells specific for TTK, LY6K, or IMP-3 were not frequently present in the patients with ESCC that weakly expressed MHC class I. Preservation of MHC class I expression on tumors might be one of the inclusion criteria for cancer vaccination with TTK, LY6K, and IMP-3 peptides. In addition to MHC class I expression, we need to consider other factors that induce and expand tumor antigen-specific T cells, including costimulatory signals by antigen presenting cells, down-regulation of antigen processing machinery, or involvement of regulatory T cells.[26]

HLA-A2402 is the most common allele in Asian people, and in Japan, it is shared by more than 60% of the population.[27] Since the expression of TTK, LY6K, and IMP-3 were frequently observed in more than 90% of ESCC, cancer vaccination with the peptides from these CTA will be applied to the majority of ESCC patients.

It has been reported that some variant cancer cells which arise from the parent tumor during tumor progression in both primary and metastatic sites can escape from TAA-specific CTLs by antigen-loss.[28–30] Since TTK, LY6K, and IMP-3 appeared to have fundamental functions for cancer cell survival,[18–20] tumor variants that have lost TTK, LY6K, or IMP-3 expression should have a critical disadvantage when surviving or proliferating. Therefore, vaccination with TTK, LY6K, and IMP-3 peptides should reduce the risk of emergence of immune escape variants, while it could induce strong antitumor immunity in patients.

In conclusion, we revealed the frequent and simultaneous expression of TTK, LY6K, and IMP-3 proteins in ESCC, and the pre-existence of antigen-specific T-cell responses to these CTA in ESCC patients. These observations strongly encouraged us to apply these CTA peptides to cancer vaccine therapy. We are currently initiating clinical vaccination trials for ESCC patients with multiepitopes involving TTK, LY6K, and IMP-3 peptides.

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