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A Novel HLA-A2–restricted CTL Epitope of Tumor-associated Antigen L6 can Inhibit Tumor Growth In Vivo

Shih-Hsin Tu,† Hsing-I Huang,‡ Su-I Lin,§ Hsin-Yu Liu,§ Yuh-Pyng Sher,‖ Sheng-Kuo Chiang,§ Pele Chong,§ Steve Roffler,¶ Guan-Chin Tseng,# Hsin-Wei Chen,§** and Shih-Jen Liu§***

Summary: Vaccines utilizing cytotoxic T lymphocyte (CTL) epitopes are promising for the treatment of cancer and chronic infectious diseases. Tumor-associated antigen L6 (TAL6) is overexpressed in some epithelial cancer cells. In this report, we detected TAL6 expression in breast cancer tissue using quantitative reverse-transcriptase-polymerase chain reaction. We found that >80% of breast tumor tissue highly expressed TAL6 compared with adjacent normal breast tissue. To identify CTL epitopes from TAL6, we synthesized 18 peptides for HLA-A2–binding assay based on the MHC-binding motif using 4 computer prediction programs. Positive binders identified by ELISA were immunized in HLA-A2 transgenic (A2 Tg) mice. Two peptides, peptide 2 and peptide 5, induced T-cell responses in A2 Tg mice. To confirm whether these peptides could be processed and presented to induce T-cell responses in vivo, A2 Tg mice were immunized with plasmid DNA encoding TAL6. We found that both peptides 2 and 5 stimulated splenocytes from TAL6-immunized mice to secrete interferon-γ. However, only peptide 5 could induce expression of the cytolytic molecule CD107a on $CD8^+$ T cells after immunization. Furthermore, peptide 5-immunized A2 Tg mice could inhibit the growth of TAL6-positive tumors (EL4/TAL6/HLA-A2) in A2 Tg mice but not in wild-type mice. These results demonstrate that the TAL6-derived CTL epitope could induce HLA-A2–restricted immunity against TAL6-expressing tumor cells.

Key Words: peptide, cytotoxic T lymphocytes, TAL6

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The tumor-associated antigen L6 (TAL6), a distant member of the transmembrane-4 superfamily (TM4SF) also known as TM4SF1, has been described as a tumor-specific antigen for different epithelial malignancies. TAL6 was originally identified with monoclonal antibody L6, which bound to human lung, breast, and colon cancer tissues but not to normal tissues.[1] As TAL6 is expressed on the surface of cancer cells, it was used as a target for antibody-based immunotherapy. In previous decades, both L6 and chimeric L6 (human-mouse) were conjugated with or without radioisotopes to treat breast cancer.[2–8] However, a phase I clinical study in patients with recurrent breast cancer using murine monoclonal antibody L6 produced only temporary remissions.[6] Similarly, a patient with aggressive, locally advanced breast cancer who was injected intravenously with radiolabeled I-131 chimeric L6 showed a partial response with transiently decreased tumor size.[5] Thus, targeting TAL with L6 monoclonal antibody has demonstrated limited therapeutic effects. The induction of T-cell immunity against TAL6 may be an alternative approach for cancer treatment.

Although TAL6 has been a target for antibody-based immunotherapy for many years, its physiological functions in cancer development are still not clear. Recently, TAL6 was found to play a role in cancer cell motility.[9] Association between TAL6 and the protease CD13 enhanced the motility of lung cancer cells.[10] Kao et al[11] reported that the expression level of TAL6 was correlated with cancer cell invasiveness. Furthermore, TAL6 is also highly expressed in the vascular endothelium of human cancers and could be a key regulator of pathologic angiogenesis in vivo.[12] Recently, TAL6 was found to be a marker for human mesenchymal stem cells[13] and to be misregulated in invasive breast cancer.[14] These studies indicate that TAL6 might play an important role in cancer metastasis.

Besides antibody therapy, induction of tumor-specific cytotoxic T lymphocytes (CTLs) is another mechanism by which to kill cancer cells. CTL-based immunotherapy for breast cancer uses breast cancer antigens that are expressed in most breast tumor cells and recognized by $CD8^+$ CTLs. Vaccination with dendritic cells (DCs) pulsed with HER-2/neu-derived peptides can induce CTL responses in patients with breast cancer.[15–17] Recently, new tumor antigen-specific CTL epitopes that could be used for immunotherapy were identified from breast cancer patients.[18–21] Furthermore, a study on measureable CTL counts and survival of breast cancer patients revealed that a reduced CTL count may be a factor or a marker of more rapid disease progression in metastatic breast cancer.[22] Another study showed that a significant fraction of breast cancer patients had stable disease after receiving up to 10 vaccinations with p53-peptide–loaded DCs at 1- to 2-week intervals.[23] These results indicate that CTLs play a major role in the progression of breast cancer.

Here, we predicted and synthesized HLA-A2–binding peptides derived from TAL6. Their binding activities were validated using ELISA-based MHC class I-binding assays. The positive binders were used to immunize HLA-A2

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transgenic mice, and CTL-cell responses were determined using the IFN-γ ELIspot assay and $CD107a^+CD8^+$ up-regulation. One of the peptides induced HLA-A2–restricted CTL responses and inhibited tumor growth in an HLA-A2 transgenic mouse model. These results provide valuable information for future cancer immunotherapy.

MATERIALS AND METHODS

Peptides

Nanomeric peptides used for the identification of CTL epitopes were selected on the basis of 4 different prediction programs from internet websites that include an HLA-binding peptide prediction (http://www-bimas.cit.nih.gov/molbio/hla_bind/),[23] SYFPEITHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm),[24] and an MHC class I-binding prediction using an artificial neural network (http://tools.immuneepitope.org/analyze/html_mhci binding20090901/mhc_binding.html),[25–27] RANPEP (http://bio.dfci.harvard.edu/RANKPEP/).[28,29] A positive control peptide, YMLDLQPETT, from the E7 protein of HPV previously identified as an HLA-A2–specific CTL epitope[30] and a negative control peptide, LYLTQDLFL, from the spike protein of SARS CoV were synthesized. Peptides (> 80% purity) were synthesized by a local company (Echo Chemical Co., Ltd. Taiwan) or at the peptide synthesis facility of the National Health Research Institutes (NHRI) in Taiwan. All peptides were dissolved in 5% DMSO at 2 mg/mL as stock solutions, stored at −20°C, and analyzed by HPLC and mass spectroscopy to verify their purity and identity.

Animals and Cell Lines

Breast cancer cell lines were obtained from the Bioresource Collection and Research Center (Hsin-Chu, Taiwan). The EL-4/TAL6 cell line was generated in Dr. Steve Roffler's laboratory (Academia Sinica, Taiwan). The EL-4/TAL6 cells were transfected with the HLA-A2 gene to generate a stable cell line EL-4/TAL6/HLA-A2 to establish a tumor model in HLA-A2 transgenic mice. The EL-4/TAL6/HLA-A2 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. Human peripheral blood mononuclear cells (PBMCs) were cultured in AIM V medium (Life Technologies, Rockville, MD). HLA-A2 transgenic mice (A2 Tg mice) were purchased from Jackson Laboratory. Specified pathogen-free mice were held at the Animal Center of NHRI. The protocols for the animal experiments in this study were approved by the Animal Committee of the NHRI.

Plasmid Construction

Plasmid pEK/HPV18E6E7, which contains an ER-targeting sequence, was designed to increase MHC class I presentation after immunization, as described in our previous report.[31] The human TAL6 DNA sequence was amplified from pcDNA3-TAL6[10] by polymerase chain reaction (PCR) using sense primer TAL6-Xba1 F primer 5′-TCTAGAATGTGCTATGGGAAG-3′ and antisense primer TAL6-Not1 R primer 5′-GCGGCCGCTTAGC AGTCATAT-3′. The PCR product was digested with *Xba*I and *Not*I restriction enzymes and then substituted for the HPV18E6E7 gene in pEK/HPV18E6E7 to create the pEK/TAL6 plasmid.

TAL6 Gene Expression in Breast Tissue

Tissue samples were obtained from 7 patients at different stages of breast cancer. Immunohistochemical analysis of tumor tissues for the expression of estrogen and progesterone receptors and human epidermal growth factor receptor 2 (Her2) was performed using standard protocol. Total RNA was isolated from breast cancer tissue and surrounding normal tissue as described in a previous report.[32] RNA (0.5 to 1 μg) was reverse-transcribed to cDNA with an oligo-dT primer in 20 μL volume using SuperScript III RT (Invitrogen, Carlsbad, CA). The human Universal Probe Library (UPL) set (Roche, Mannheim, Germany) was used to perform the real-time qPCR assay for gene expression in isolated cell populations. The specific primers and UPL number were as follows: GAPDH, 5′-tccactg gcgtcttcacc-3′ (forward) and 5′-ggcagagatgatgacccttt-3′ (reverse) with UPL#45; TAL6, 5′-tttctggcatcgtaggaggt-3′ (forward) and 5′-ccagcccaatgaagacaaat-3′ (reverse) with UPL#51. The reaction mixture contained 2 μL of cDNA for QPCR, 0.2 μM primers, and LightCycler 480 Probe Master (Roche), and the reaction was carried out in a LightCycler 480 system (Roche). All qPCR reactions were carried out with an initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, at 60°C for 20 seconds, and at 72°C for 2 seconds. Target gene expression was calculated using the comparative method for relative quantity upon normalization to GAPDH gene expression.

Immunohistochemical Staining

Formalin-fixed paraffin-embedded breast cancer specimens were obtained from 22 patients under surgery at China Medical University Hospital. This study was approved by the Institutional Review Committee of China Medical University Hospital. Four-micrometer-thick sections were cut and immunostained for human TAL6 using a rabbit anti-human TAL6 antibody (Sigma), biotinylated goat anti-rabbit antibody, horseradish peroxidase-conjugated avidin-biotin complex (ABC) from Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), and AEC chromogen (Vector Laboratories). The sections were counterstained with hematoxylin and mounted. The expression levels of TAL6 were assessed by a pathologist.

In vitro Peptide-binding Assay

The in vitro ELISA-based peptide-binding assay was modified according to a previous report.[31] In brief, a 96-well microtiter plate was incubated overnight with 5 μg/mL of anti-HLA antibody (W6/32) in 100 mM carbonate buffer, pH 9.6, to coat the plate. After blocking with 10% nonfat milk/PBS, the concentrated refolded HLA/peptide complexes were diluted with 2% BSA and added to the plate for 1 hour. HRP-conjugated rabbit anti-human β2-microglobulin (1:2500) was used to detect complex formation. The enhancing reagent EnVision-HRP (Dako, Denmark) was used to amplify the signal. The substrate 3, 3′, 5, 5′-tetramethylbenzidine was used for color development, which was stopped with 1 N H_2SO_4. Absorbed density (OD) was measured at 450 nm using an ELISA reader (Molecular device, Sunnyvale, CA).

Immunization of HLA-A2 Mice

One milligram of the indicated 9-mer peptides and 1 mg of a universal Th epitope Pan-DR epitope peptide (PADRE: AKFVAAWTLKAAA) in 0.5 mL PBS were mixed with 0.5 mL of incomplete Freund's adjuvant (IFA). The positive control peptide for HLA-A2 was the HPV16 E7-derived peptide YML (YMLDLQPETT), and the negative control peptide LYL (LYLTQDLFL) was from

SARS CoV spike protein. One hundred microliters of the mixture was injected subcutaneously (SC) into the tail base of A2 Tg mice twice at a 7-day interval. The spleen cells were harvested 7 days after the second injection and cultured with 10 μg/mL of peptide for the IFN-γ–secreting ELISPOT assay.

For DNA immunization, A2 Tg mice were injected intramuscularly with 100 μg of pEK/TAL6 or control plasmid (pCIneo) every 3 weeks. On day 7 after the second injection, splenocytes were harvested, and T-cell responses were measured using the methods described later.

ELISPOT Assay

The ELISPOT assay was performed as previously described.[10] In brief, 2×10^5 and 5×10^5 spleen cells with 10 μg/mL of the indicated peptides were added to a 96-well PVDF-membrane plate coated with anti-IFN-γ antibody. The plates were then incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 hours. After the incubation period, the cells were removed by washing the plates 6 times with 0.05% (w/v) Tween 20 in PBS. A 50 μL aliquot of biotinylated secondary anti-IFN-γ antibody (clone R46A2; eBioscience, San Diego, CA) was added to each well. After 2 hours of incubation the plate was washed, and streptavidin-HRP (eBioscience) was added. Spots were developed using a 3-amine-9-ethyl carbazole (ACE, Sigma) solution. The reaction was stopped after 4 to 6 minutes by running the plate under tap water. The spots were then counted using an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

Peptide-specific T-cell Immunity in Breast Cancer Patients

For the collection of blood samples from breast cancer patients, informed consent was obtained from each patient according to the Declaration of Helsinki. The operation protocol was approved by the Institutional Review Board of the NHRI. Twenty milliliter of blood samples were collected from patients in heparin tubes. Two milliliters of blood was aliquoted for HLA typing, and the rest of the blood was used to prepare PBMCs by Ficoll-Hypaque centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ) at 800*g* for 30 minutes. PBMCs were stored in liquid nitrogen until use. For the IFN-γ ELISPOT assay, PBMCs (5×10^5/well) were added to 96-well PVDF-membrane plates in the presence of peptides (10 μg/mL). After incubation for 48 hours, IFN-γ–secreting T cells were detected using HRP-conjugated streptavidin and ACE as shown previously. The reaction products were detected using an ELISPOT reader. The mean number of spots was from triplicate experimental wells.

CD107 Cytotoxicity Assay

CD107 assay was described as an alternative cytotoxicity assay in previous reports.[33–35] In brief, A2 Tg mice were injected SC with the indicated peptides (50 μg/mL) emulsified in IFA in the presence of 50 μg PADRE peptide. On day 7 after the second immunization, splenocytes were harvested and then resuspended to 2×10^7 cells/mL in medium that contained 10 μg/mL of the indicated peptides and PE-conjugated anti-mouse CD107a antibody (1:100) in 96-well round-bottom plates. After 2 hours of incubation at 37°C, brefeldin A (10 μg/mL) and monensin (0.66 μg/mL) were added for another 6 hours. The plates were washed with PBS containing 0.1% fetal bovine serum, and anti-mouse Fc antibody (1:100) was added for 5 minutes, followed by addition of the FITC-conjugated anti-mouse CD8 antibody (1:100) for 30 minutes. The cytotoxic $CD107a^+CD8^+$ cells were analyzed on a fluorescence-activated cell sorter (FACScalibur, BD Bioscience).

Chromium Release Assay

The A2 Tg mice (6 to 8 wk old) were immunized twice SC at a 7-day interval through the footpad with peptides (50 μg/mouse) that were formulated with ISA and CpGODN (10 μg/mouse). Seven days after the last immunization, splenocytes (1×10^6 cells/mL) were cultured in vitro with 10 μg/mL of indicated peptides and 10 U/mL of recombinant human IL-2 in 24-well plates for 5 days. After incubation, MCF-7 or MCF-7/TAL6 cells (5×10^5) were labeled with 100 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$; PerkinElmer, Waltham, MA) at 37°C for 1 hour to serve as target cells. The peptide-stimulated splenocytes were mixed with labeled target cells at various effector-to-target ratios as indicated. After a 4-hour incubation at 37°C, the radioactivity of the supernatant was measured using a gamma counter (PerkinElmer). The percentage of specific lysis was calculated using the following equation: $100 \times [(\text{Experimental release} - \text{Spontaneous release})/(\text{Maximal release} - \text{Spontaneous release})]$.

Animal Study

A2 Tg mice were injected SC twice at a 2-week interval with peptides formulated in IFA with 50 μg of PADRE. Seven days after the second immunization, the EL-4/TAL6 or EL4/TAL6/HLA-A2 cells (2×10^5) were inoculated SC on the opposite site of the peptide injection. Tumor sizes were measured 3 times per week. Tumor volume was calculated using the formula: Tumor volume = Length × Width × Width/2.

Statistical Analysis

The statistical significance of differences between the mean values of the experimental groups was determined using the Student *t* test. The differences were considered statistically significant if the *P* value was < 0.05.

RESULTS

TAL6 Expression in Breast Cancer Cell Lines and Tissues

Previous reports have revealed that TAL6 antigen can be detected in nearly 80% of breast cancer tissues in the United States using monoclonal antibody L6.[36,37] Using flow cytometry, we found that TAL6 was expressed on BT474, MDA-MB-453, and MDA-MB-468 breast cancer cell lines but not on MCF-7 cells (Fig. 1A). After transfection of MCF-7 cells with the TAL6 gene to generate the MCF-7/TAL6 cell line, we could detect the expression of TAL6 on the surface of MCF-7/TAL6 cells. To confirm TAL6 expression in breast cancer patients in Taiwan, we collected specimens from cancer tissues and their adjacent normal tissues (Table 1). TAL6 expression levels were determined by quantitative real-time PCR. We found that > 90% (6 of 7) of breast cancer tissues overexpressed TAL6 relative to adjacent normal breast tissue (Fig.1B). Moreover, we collected breast cancer tissues and analyzed TAL6 expression using immunologic staining. The TAL6 protein was detected in around 80% of the 22 samples analyzed, although at various levels (Fig. 1C). These results suggest that breast cancer tissues from 2 different geo-

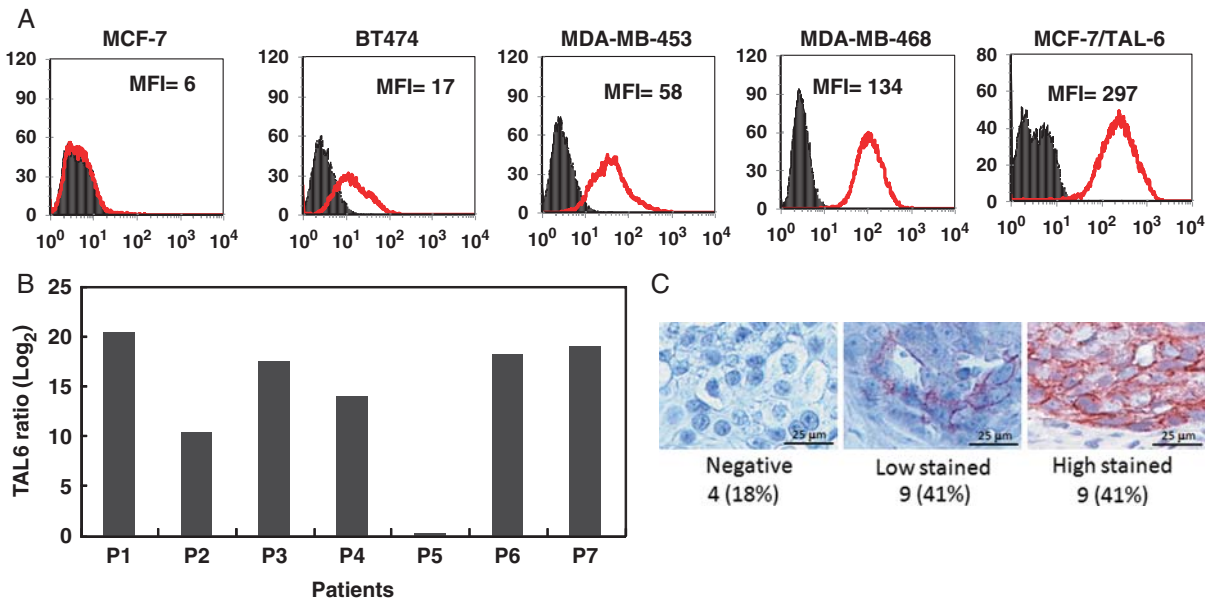

FIGURE 1. TAL6 expressed in breast cancer cell lines and tissues. A, The expression of TAL6 was determined by flow cytometry using a mouse anti-TAL6 antibody (solid line) or an isotype control antibody (shaded) and then probed with goat anti-mouse FITC-conjugated antibody. B, The breast cancer tissue and the adjacent normal tissues were analyzed for TAL6 RNA transcripts. The RNA transcripts of NADH were used as an internal control. The data are expressed as the ratio of RNA transcripts in cancer/normal tissue (Log2). C, Breast cancer tissues were stained with anti-TAL6 antibody, and positive samples were confirmed by the pathologist.

graphic regions (United States and Taiwan) express TAL6 at similar frequencies.

Peptide Binding to HLA-A*0201 Molecules

Several MHC-binding peptide prediction algorithms have been developed to assist the identification of novel T-cell epitopes. The MHC-binding motif prediction programs are based on the dissociation rates of MHC/peptide complexes,[38] peptide sequence similarity with well-known MHC-binding peptides,[24] algorithms of the artificial neural network,[25,26] and position-specific scoring matrices or profiles (RANKPEP).[28,29] As the RANKPEP program takes into account possible proteasomal cleavage sites within the predicted peptides, we selected 14 peptides on the basis of this program. The other 4 peptides were selected from the top 10 binders from 3 other prediction methods that failed using the RANPEP method. A total of 18 peptides (Table 2) were synthesized from human TAL6. We used an ELISA-based system to evaluate their binding ability to the HLA-A*0201 (HLA-A2) molecule.[31] In this system, we coated 96-well plates with anti-HLA monoclonal antibodies, exposed the plates to refolded HLA-A2 complexes, and then detected bound HLA-A2 complexes with an HRP-conjugated anti-β2-microglobulin monoclonal antibody. A positive control peptide YML (YMLDLQPETT, from the E7 protein of HPV), previously identified as an HLA-A2–specific CTL epitope,[30] and a negative control peptide LYL (LYLTQDLFL, from the spike protein of SARS CoV) were included in this experiment. The relative binding activity to the positive control peptide was arbitrarily set as 100%. Nine experimental peptides with relative binding activities higher than the positive control were selected for further study (Table 2).

Peptide-specific T-cell Responses Induced in HLA-A2 Transgenic Mice

To examine the T-cell reactivity of the peptides in vivo, the 9 peptides with the highest MHC-binding activity were selected to immunize A2 Tg mice. For each peptide, 50 μg of peptide was formulated with 50 μg of PADRE peptide in IFA. HLA-A2 mice were immunized SC on days 0 and 7. Splenocytes were harvested 7 days after the final immunization, and T-cell reactivity was analyzed using the IFN-γ–secreting ELISPOT assay. Only peptide 2 (SLVGLALLC) and peptide 5 (LLMLLPAFV) induced substantial INF-γ secretion (Fig. 2A). Although these peptides could induce T-cell responses in HLA-A2 transgenic mice, the other important question was whether they could be processed in vivo. To answer this question, we designed a mammalian expression vector that contained an ER-targeting sequence to increase the presentation efficiency.[31] We generated the plasmid pEK/TAL6 by inserting a human TAL6 sequence in place of the HPV18 E6E7 sequence in pEK/HPV18E6E7. Mice were immunized with pEK/TAL6 2 to 3 times at

TABLE 1. Characteristics of the Breast Cancer Patients

Patient No.	Age	Cancer Stage	Receptor Status		
			ER (%)	PR (%)	Her2
P1	81	IIIA	> 95	30	0
P2	44	0	95	5	3+
P3	52	IIIC	> 95	15	1+
P4	56	I	—	—	—
P5	52	IIB	60	90	2+
P6	70	IIIC	95	1	3+
P7	48	I	95	> 95	3+

ER indicates estrogen receptor; Her2, human epidermal growth factor receptor 2; PR, progesterone receptor.

AQ8 **TABLE 2.** HLA-A2-binding Peptides Derived From TAL6 Were Predicted Using Different Algorithms

Peptides	Amino Acid Start Position	Sequence	Score of Parker's Method*	SYFPEITHI†	ANN (IC50 nM)‡	RANKPEP§	Relative Binding Ability (%)‖
1	9	CIGHSLVGL	6.8	25	>300	63	132.4
2	13	SLVGLALLC	<6.0	<21	>300	61	125.7
3	18	ALLCIAANI	<6.0	24	78.2	<59	92.0
4	53	IVGGGLLML	7.3	25	>300	63	75.1
5	58	LLMLLPAFV	2406.1	26	8.7	<59	166.5
6	60	MLLPAFVFI	685.8	24	8.1	100	179.8
7	89	AMLSSVLAA	30.5	22	57.2	97	121.2
8	90	MLSSVLAAL	83.5	27	20.6	103	160.4
9	93	SVLAALIGI	7.8	21	207.4	100	179.1
10	114	GLAEGPLCL	87.6	28	86.9	66	99.6
11	154	HIVEWNVSL	<6.0	24	200.9	71	120.6
12	161	SLFSILLAL	181.8	29	14.2	111	85.1
13	164	SILLALGGI	<6.0	25	>300	71	63.9
14	168	ALGGIEFIL	50.8	24	81.5	<59	114.0
15	173	EFILCLIQV	<6.0	<21	>300	59	ND
16	174	FILCLIQVI	23.0	24	57.1	<59	ND
17	177	CLIQVINGV	160.0	28	48.6	92	39.0
18	181	VINGVLGGI	<6.0	25	>300	65	58.6

*The score is the estimation of the half-time peptide disassociation from HLA-A2 molecule based on the method of Parker.[38]
†The score is the probability of a sequence processed and presented to HLA-A2 molecule.[24]
‡The peptide-binding affinity is predicted by the artificial neural networks (ANN) approach.[25,27]
§This program predicts MHC-peptide binders from protein sequence using Position Specific Scoring Matrices and a C-terminal end that is likely to be cleaved by proteasome.[28,29]
‖The relative binding activity is calculated relative to that of a positive control peptide (YMLDLQPETT, an HLA-A2–restricted peptide derived from HPV E7) set as a reference, as described in the Materials and methods section.
ND indicates not determined.

3-week intervals as described in the Materials and methods section before killing them 7 days after the final boost. Peptide-specific T-cell responses were determined using the IFN-γ ELISPOT assay. Fig. 2B shows that the internal positive control peptide SII (SIINFEKL, an H-2K^b-restricted epitope derived from ovalbumin) and peptide 2 and 5 could stimulate T cells to secrete INF-γ but that immunization with control vector pCIneo did not. These results demonstrate that peptide 2 and 5 could be processed from the whole protein and presented to induce CTL responses in vivo.

Identification of CTL Epitopes in Breast Cancer Patients

To determine whether these CTL epitopes (peptide 2 and 5) could be presented and induce IFN-γ production in humans, we collected PBMCs from HLA-A2–positive breast cancer patients. The PBMCs were ex vivo stimulated with the CTL peptides 2 and 5. After stimulation for 48 hours, the IFN-γ production spots were counted. Peptides 2 and 5 stimulated IFN-γ secretion in PBMCs from patients 30 and 35 but not in those from other HLA-A2–positive breast cancer patients (Fig. 3.) The nonreactivity of PBMCs may be because of the low frequency of CTL precursor cells in these patients. These results indicate that human cells could present both TAL6-derived peptides 2 and 5 epitopes, and that peptide-specific T cells also existed in some HLA-A2–positive breast cancer patients.

TAL6-derived CTL Epitopes Can Suppress Tumor Growth in HLA-A2 Transgenic Mice

To directly determine whether both peptides could induce cytolytic activity in A2 Tg mice, splenocytes from peptide-immunized A2 Tg mice were restimulated with peptide 2 or 5 in vitro. The expression of CD107a molecules on CD8$^+$ T cells was analyzed to determine their cytolytic activity. It is interesting to note that we found that peptide 5 could induce a higher number of CD107a$^+$/CD8$^+$ cells (0.35 ± 0.09 vs. 0.15 ± 0.03) compared with peptide 2 (0.17 ± 0.03 vs. 0.15 ± 0.03) (Fig. 4). Because of the ex vivo cytolytic activity induced by peptide 5, it was of interest to investigate whether peptide 5 could be used to treat TAL6-expressing cancer in vivo. We established a cell line (EL4/TAL6/HLA-A2) that expressed both TAL6 and HLA-A2. This cell line grew rapidly in wild-type C57BL/6 and HLA-A2 transgenic mice. Therefore, we immunized A2 Tg mice twice with peptide 2 or 5 in IFA in the presence of a Th epitope peptide (PADRE). EL4/TAL6/HLA-A2 cells were inoculated 7 days after the second immunization. Tumor growth was suppressed in mice immunized with peptide 5 but not peptide 2 or control peptide (Fig. 5). The protective effect of peptide 5 was not observed in wild-type mice inoculated with EL4/TAL6 tumor cells. These results indicate that peptide 5 immunization can induce HLA-A2–specific antitumor effects in vivo.

TAL6-derived CTLs Kill TAL6-expressing Human Breast Cancer Cells

Although the induction of CTL responses by peptide 5 could inhibit the growth of mouse tumor cells that expressed both HLA-A2 and TAL6, their ability to directly kill human cancer cells remained to be established. To prove that peptide 5-induced CTLs in A2 Tg mice were able to kill human breast cancer cells, we established an MCF-7/TAL6 stable cell line as a model. The parent MCF-7 cells express the HLA-A2 haplotype; it does not produce TAL6. Peptide 5 was used to immunize A2 Tg mice twice, and the isolated splenocytes were restimulated with 10 μg/mL of peptide 5 or with an irrelevant peptide for 5 days. The peptide 5-induced CTL could not kill parental MCF-7 cells

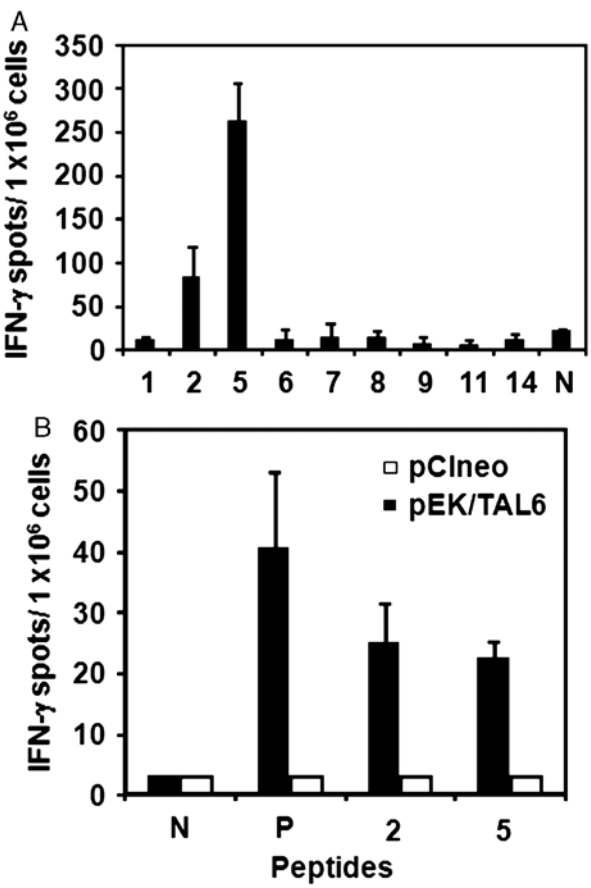

FIGURE 2. Peptide and DNA immunization induces HLA-A2-specific T-cell responses. A, HLA-A2 transgenic mice were immunized SC twice with various peptides formulated with the Th epitope peptide PADRE in incomplete Freund's adjuvant (IFA). Seven days after the final immunization, splenocytes (5×10^5 cells/well) were incubated with 10 μg/mL of peptide in anti-IFN-γ–coated ELISpot plates for 48 hours at 37°C. Spots were developed and evaluated in an ELISpot reader using computer-aided calculation software. Numbers represent mean values of 3 independent experiments. B, For DNA immunization, A2 Tg mice were immunized with 100 μg/mL of plasmid pEK/TAL6 or control plasmid pCIneo, intramuscularly. Seven days after the second immunization, splenocytes were removed and stimulated with peptide to detect T-cell responses. The IFN-γ ELISPOT assay was performed to detect IFN-γ–secreting cells. IFN indicates interferon; N, negative control peptide; P, positive control peptide.

but did kill MCF-7/TAL6 cells (Fig. 6). These data show that peptide 5 immunization of A2 Tg mice induces functional cytotoxic T cells that can kill human breast cancer cells that express TAL6.

DISCUSSION

The TAL6 antigen has been a target for antibody-based immunotherapy of breast cancer for many years.[3,5,39,40] The lack of clinical responses to antibody-based therapy may be because of the antibody half-life or because of its low targeting efficiency in vivo. More importantly, the lack of NK cell-DC crosstalk enhancement of cellular immunity triggered by antibody-based therapy may limit its efficacy.[41] The induction of T-cell–mediated immunity provides the other arm of tumor therapy. However, the development of CTL-based immunotherapy using TAL6 as an attractive target for cancer treatment has not been reported. To our knowledge, this is the first report identifying an HLA-A2–restricted antigenic peptide (LLMLLPAFV) from the TAL6 antigen for potential CTL-based immunotherapy. We also demonstrated that immunization of HLA-A2 transgenic mice with this peptide formulated in IFA could inhibit TAL6-expressing tumor growth in vivo. Although peptide/HLA-A2 complexes recognized by murine T cells of A2 Tg mice and human T cells might be different as a result of the antigen presentation machinery, several investigators have identified HLA-A2–restricted CTL epitopes using the A2 Tg mouse model that are recognized in human T cells as well.[30,42,43] These data suggest that the A2 Tg mouse model is useful in the identification of human CTL epitopes.

Technologies for the identification of novel CTL epitopes have been described in our previous report.[31] To improve the efficiency of peptide selection and avoid bias from using a single MHC-binding prediction algorithm, a total of 18 peptides (9-mers) derived from the TAL6 antigen were synthesized on the basis of several MHC class I peptide-binding prediction algorithms. The selected peptides were refolded with HLA-A2 and β2-microglobulin, and the peptide/MHC complexes were detected using a cell-free ELISA-based binding assay. The potential CTL epitopes (peptides 2 and 5) could also stabilize HLA-A2 complexes using a conventional T2 cell-binding assay (Supplemental Figure 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A193). We found that peptide 2 (SLVGLALLC) was ranked in the middle when the RANPEP peptide-binding prediction algorithm was used but was in the low-binding category when other peptide-binding prediction algorithms were used (Table 2). It is interesting to note that peptide 2 not only could induce T-cell responses after immunization of A2 Tg mice but was also processed in vivo after DNA immunization of A2 Tg mice (Fig. 2). However, peptide 2 immunization of A2 Tg mice could neither generate cytotoxic T cells ($CD107a^+CD8^+$) nor inhibit tumor growth (Figs. 4, 5). In contrast, peptide 5 (LLMLLPAFV) was ranked as the highest HLA-A2 binder on the basis of

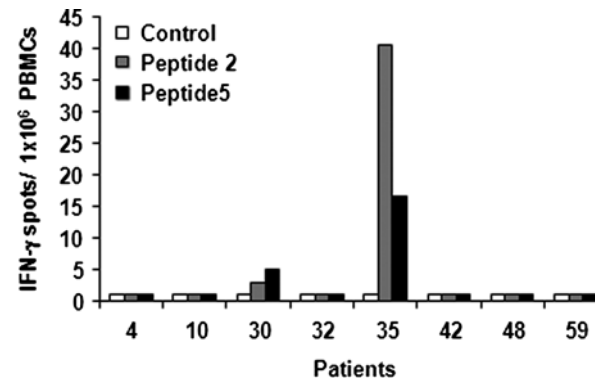

FIGURE 3. Peptide-stimulated PBMCs derived from breast cancer patients secrete IFN-γ. PBMCs (5×10^5/well) from HLA-A2–positive breast cancer patients were incubated with 10 μg/mL of negative control peptide LYL or with experimental peptides 2 and 5. The IFN-γ–secreting spots were developed and analyzed using an ELISpot reader. The data are presented as the means of triplicate determinations. A reaction was determined to be positive if the mean number of spots was greater than twice the average number of spots determined with the negative control. IFN indicates interferon.

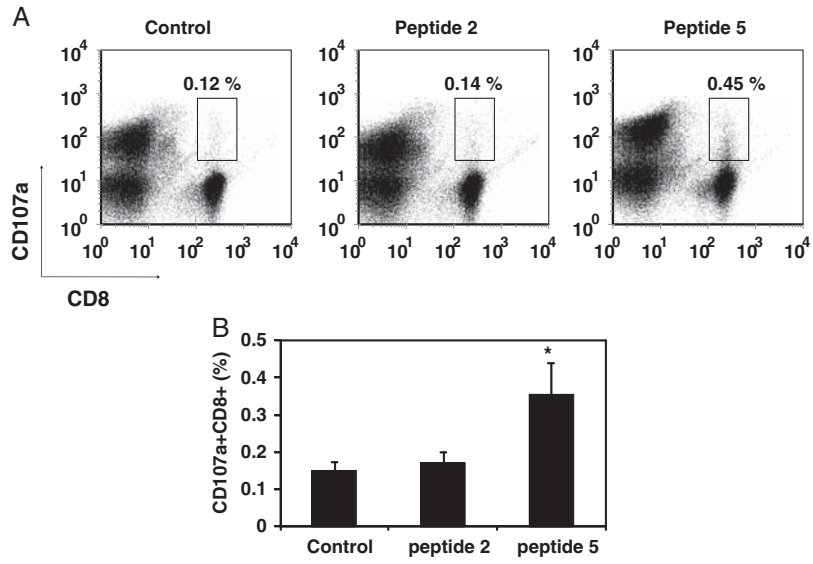

FIGURE 4. Peptide immunization of HLA-A2 transgenic mice induces cytotoxic T lymphocytes. Transport of the lysosomal protein CD107a to the cell surface was used as a marker for cytotoxic T lymphocytes. Splenocytes harvested from peptide-immunized mice were stimulated with 10 μg/mL of peptide 2 or 5 for 18 hours in the presence of PE-conjugated anti-CD107a. After stimulation, FITC-conjugated anti-CD8 antibody was used to detect $CD8^+$ T cells. The percentage of $CD107a^+CD8^+$ cells in each immunized group was determined by flow cytometry. B, Results obtained from 3 mice are expressed as mean ± SD (*$P < 0.01$).

Parker algorithm software, but its binding ability to HLA-A2 was predicted to be low using the RANPEP algorithm (Table 1). This peptide not only could be processed in vivo to induce T-cell responses after DNA immunization (Fig. 2) but also induced peptide-specific cytotoxic T cells ($CD107a^+CD8^+$) (Fig. 4). Furthermore, peptide 5 immunization could inhibit EL4/TLA6/HLA-A2 tumor cell growth in A2 Tg mice (Fig. 5). These results demonstrate that peptide 5 immunization can induce HLA-A2–restricted TAL6-specific antitumor immunity. Although DNA immunization could induce peptide 2-specific T cells to secrete IFN-γ, it did not induce cytotoxic T cells to kill tumor cells in vivo. These results are similar to those of a previous report, in which a peptide derived from NS3 of HCV could stimulate T cells to secrete IFN-γ but did not elicit cytotoxic activity after DNA immunization[44] and also showed a $CD8^+$ T cell line from NS4 of HCV that could stimulate T cells to secrete IFN-γ but which also lacked cytotoxic activity.[45] These noncytolytic T cells have been shown to be beneficial in killing HBV or HCV but not infected cells.[46,47] For cancer immunotherapy, the noncytolytic T cells did not provide protective effects in vivo (Fig. 5A).

These studies indicate that TAL6 is not only a tumor marker but might also play a role in cancer metastasis. In this study, we found that EL4/TAL6/HLA-A2 is a very aggressive tumor cell line in mice. The peptide 5 immunization could not eradicate tumor growth in vivo completely. To enhance the antitumor effect of peptide 5 immunization, immunostimulatory adjuvants may have to be included in the final vaccine formulation. In the future, we will use peptide 5 formulated with adjuvants or pulsed DCs to immunize A2 Tg mice and then adoptively transfer

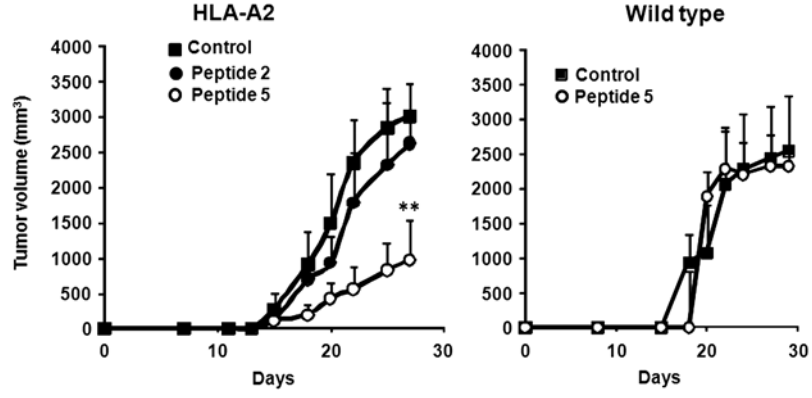

FIGURE 5. Peptide immunizations of A2 Tg mice induces antitumor responses. A2 Tg mice (A) or C57BL/6 wild-type mice (B) were immunized with peptide/IFA (50 μg/mouse) in the back of the left flank subcutaneously twice at 2-week intervals. After 21 days, EL4/TAL6/HLA-A2 (A) or EL4/TAL6 (B) cells (2×10^5) were inoculated SC at the abdominal site. Tumor size was monitored every 2 to 3 days until tumor volume exceeded 3 cm^3. **$P < 0.005$ compared with PBS-immunized control mice (A2 Tg group, n = 10; C57BL/6 wild-type group, n = 6). TAL6 indicates tumor-associated antigen L6.

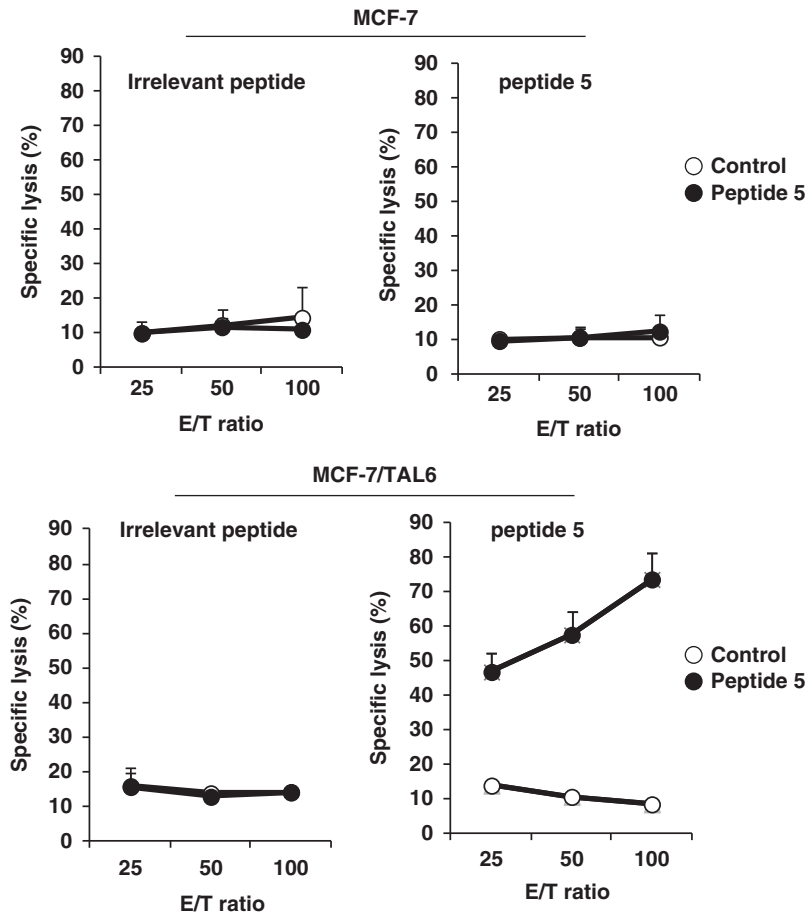

FIGURE 6. Peptide immunizations of A2 Tg mice induce CTLs against human breast cancer cells. A2 Tg mice were immunized with peptide/IFA (50 μg/mouse) subcutaneously in the footpad twice at 7 days interval. Splenocytes were isolated and stimulated with peptide 5 or irrelevant peptide (10 μg/mL) and human IL-2 (10 U/mL) for 5 days as effector cells. Both MCF-7 and MCF-7/TAL6 cells (5×10^6) were labeled with chromium (100 μCi) for 1 hour as target cells. The target cells (5×10^3/well) were incubated with different ratios of effector cells (1:25, 1:50, 1:100). Specific lysis (%) was calculated as: (Sample release (cpm) − Spontaneous release (cpm))/(Maximum release (cpm) − Spontaneous release (cpm)) × 100.

$CD8^+$ T cells from immunized mice into human breast cancer cell-bearing SCID mice. Alternatively, the combination of multiple CTL epitopes from other tumor antigens (ie, p53, erbB2, CEA) in a single formulation may further enhance their antitumor activity in future applications. The identification of CTL epitopes for immunotherapy may provide other mechanisms to inhibit tumor growth beyond antibody-based therapy.

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CONFLICTS OF INTEREST/FINANCIAL DISCLOSURES

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