Identification of a Promiscuous Epitope Peptide Derived From HSP70

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Summary: We previously found that heat-shock protein 70 (HSP70) is expressed on hepatocellular carcinoma cells and developed an HSP70 mRNA-transfected dendritic cell therapy for treating unresectable or recurrent hepatocellular carcinoma. The phase I trial was completed successfully. The purpose of this study is to identify a promiscuous epitope peptide derived from HSP70 for the purpose of developing a novel cancer peptide vaccine. Using a computational algorithm to analyze the specificity of previously reported major histocompatibility complex class I–binding peptides, we selected candidates that bound to > 2 of the 3 HLA types. Twenty-nine HSP70-derived peptides (9-mers) that bound to HLA-class I was selected. The peptides were prioritized based on the results of peptide binding experiments. Using dendritic cells stimulated with the candidate peptide described previously as stimulators and CD8+ T cells as effectors, an ELISPOT assay was performed. Cytotoxicity of CD8 lymphocytes stimulated with the candidate peptides toward HSP70-expressing cancer cells was analyzed using an xCELLigence System. Peptides were administered to HLA-A 24 transgenic mice as vaccines, and peptide-specific T-cell induction was measured in vivo. We identified a multi-HLA-class I–binding epitope peptide that bound to HLA-A*02:01, *02:06, and *24:02 in vitro using an interferon-γ ELISPOT immune response induction assay. Cytotoxicity was confirmed in vitro, and safety and immune response induction were confirmed in vivo using HLA-A 24 transgenic mice. Our study demonstrated that the promiscuous HSP70-derived peptide is applicable to cancer immunotherapy in patients with HLA-A*24:02-positive, *02:01-positive, and *02:06-positive HSP70-expressing cancers.

Unresectable or recurrent hepatocellular carcinoma (HCC) is associated with poor prognosis. With the exception of sunitinib, there are no established systemic antitumor therapies. Recently, immune checkpoint inhibitors have become available for the treatment of HCC and the results of phase I/II trials of nivolumab, an antiprogrammed cell death-1 antibody, showed good response in some patients, but the majority of the patients remain refractory.[1,2] In contrast, despite the fact that the recurrence rate is still high even after curative resection of HCC, postoperative adjuvant therapy has not been established.[3] Proteomic and immunohistochemical staining experiments in our department demonstrated that heat-shock protein 70 (HSP70) is highly and specifically expressed by HCC cells.[4,5] On the basis of these results, we developed an HSP70 mRNA-transfected dendritic cell (DC) therapy for treating unresectable or recurrent HCC, and a phase I trial confirming the safety and efficiency of this therapy has been completed.[6] As this cell-based therapy is complex and therefore suitable for only a limited number of facilities, in the present study, we developed a novel peptide vaccine-based immunotherapy by identifying the functional epitope peptide in the HSP70 mRNA DC therapy using a computational algorithm and patient-derived peripheral blood mononuclear cells (PBMCs).[7]

The HLA-A gene with the highest frequency in Asian populations, especially in the Japanese, is HLA-A*24:02, whereas the frequency of this gene is low in Caucasians.[8] In contrast, the gene frequency of HLA-A*02:01 is high among a variety of ethnic groups, including both Asians and Caucasians.[9] These findings suggest that HSP70-derived cytotoxic T lymphocyte (CTL) epitopes that bind to both HLA-A*24:02 and A*02:01 would be highly useful in immunotherapies aimed at treating HCC. In the present study, we identified a human HSP70-derived epitope peptide that binds to HLA-A*24:02, A*02:01, and *A02:06.

MATERIALS AND METHODS

Patients

Fourteen HLA-A*24:02-positive, *02:01-positive, or *02:06-positive patients who received the DC-based therapy participated in the clinical trial (UMIN000010691).

Selection of Candidate Peptides

To select candidate peptides derived from HSP70, we used a peptide prediction system developed by the NEC Corporation. The system employs a committee-based framework of query learning using hidden Markov models as the component algorithm.[7] The system enables comprehensive searching of a large number (20^9) of peptides in a small number of experiments. Learning was achieved over 7 rounds of feedback loops, in which our computational algorithm was used to determine the next set of peptides to be analyzed based on the results of the previous iterations. After these training cycles, the algorithm provided a real number prediction of major histocompatibility complex binding peptides with high accuracy. Using this prediction system, we selected a total of 17 candidate peptides (Table 1).

Peptides

HSP70-derived 9-mer peptides that exhibited high binding affinity to HLA-A*24:02, 02:01, and 02:06 were synthesized by Scrum (Tokyo, Japan) and purified by HPLC to purity of >90%. The epitope peptides HIV-*A24:02 (RYLRDQQLL), HIV-A*02:01 (SLYNTVATL), and HIV-*A02:06 (ATLEEMMTA) were synthesized as negative controls.[10–12]

Peptide Binding Assay

Binding of peptides to HLA-A*24:02 was examined using acid stripping and a reconstitution assay, as previously described by Zeh et al,[13] with minor modifications. Briefly, C1R-A24 cells were exposed to citrate-phosphate buffer (pH 3.3) and then reconstituted with graded concentrations of peptide and 0.1 μM human β2-microglobulin (M-4890; Sigma, St Louis, MO) in DMEM containing 0.25% bovine serum albumin. Fluorescein isothiocyanate–labeled monoclonal antibody 17A12 (Tahara and colleagues) was used to detect properly folded and peptide-bound HLA-A*24:02 molecules.[14] Fluorescence intensity was measured using a FACScan instrument (Becton-Dickinson Japan, Tokyo, Japan). Both high binding and low binding peptides (*HER2-63* TYLPTNASL and *Met149* RVWE-SATPL, respectively) were included in each assay, and their binding was used to normalize variations between experiments. The affinity of each peptide was calculated as previously described (Udaka and colleagues). A similar method was used to assess the binding of peptides to HLA-A*02:01 and HLA-A*02:06, and details will be published elsewhere.[15]

Separation of Adherent and Nonadherent Cells

PBMCs were harvested from patients enrolled in the clinical trial described previously using a COBE Spectra Apheresis System (COBE BCT Inc., Lakewood, CO). PBMCs from 3000 mL of blood were enriched by density gradient centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). The PBMCs were incubated for 45 minutes in a 5% CO_2 atmosphere at 37°C in serum-free AIM-V medium (Gibco, Paisley, Scotland). Plastic-adherent cells were used to generate DCs, whereas nonadherent cells were used to generate CTLs.

Generation of DCs

DCs were generated as described previously.[6] Briefly, plastic-adherent cells were cultured in AIM-V medium containing 800 U/mL of granulocyte-macrophage colony-stimulating factor (Osteogenetics GmbH, Wurzburg, Germany) and 500 U/mL of interleukin (IL)-4 (Osteogenetics GmbH). On day 6, immature DCs were cultured in AIM-V medium containing 300 U/mL of tumor necrosis factor-α (R&D Systems, Minneapolis, MN). Cultures were checked for endotoxins and contamination with *Mycoplasma* and other bacteria before administration. On day 10, floating and loosely adherent cells were collected as mature DCs.

Preparation of CD8+ T Lymphocytes

Nonadherent cells were cultured in AIM-V medium. On days 3, 5, and 7, recombinant human IL-2 (Shionogi Pharmaceutical Co., Tokyo, Japan) was added to the cultures to a final concentration of 10 IU/mL. The plates were incubated in a 5% CO_2 atmosphere at 37°C. On day 10, cultured cells were collected and washed 3 times with phosphate buffered saline (PBS). T cells were then isolated by MACS depletion (Miltenyi Biotec, Bergisch Gladbach,

TABLE 1. Candidate Epitope Peptides Derived From HSP70

Peptide	Position	Amino Acid Sequence	log Kd		
			A*24:02	A*02:01	A*02:06
Y44	470	GVPQIEVTF	−6.45	>−3	−5.13
Y45	204	TFDVSILTI	−6.23	>−3	>−3
Y46	114	FYPEEISSM	−7.66	>−3	>−3
Y47	348	KLLQDFFNG	>−3	−4.96	−5.27
Y48	**335**	**VLVGGSTRI**	**−6.17**	**−4.86**	**−5.07**
Y49	**122**	**MVLTKMKEI**	**−5.81**	**−4.59**	**−5.53**
Y50	**371**	**YGAAVQAAI**	**−5.50**	**−4.9**	**−5.37**
Y51	173	INEPTAAAI	>−3	>−3	>−3
Y52	392	LLLDVAPLS	>−3	−6.06	−6.17
Y68	**448**	**AMTKDNNLL**	**−5.49**	**−5.56**	**−4.28**
Y69	**297**	**ITRARFEEL**	**−5.20**	**−4.33**	**−5.58**
Y70	454	NLLGRFELS	>−3	−4.56	−5.18
Y71	329	AQIHDLVLV	>−3	−6.15	−6.46
Y72	59	VALNPQNTV	>−3	−4.66	−4.06
Y73	545	YAFNMKSAV	>−3	−5.09	−5.02
Y74	434	NQPGVLIQV	>−3	−5.91	−6.42
Y75	**138**	**SVTNAVITV**	**−4.37**	**−4.76**	**−5.59**

Bold values indicate candidate peptides.
HSP70 indicates heat-shock protein 70.

Germany). Cultured cells were labeled with microbeads using a CD8 T-cell isolation kit and separated on magnetic columns in a VarioMACS separator, according to the manufacturer's recommendations (Miltenyi Biotec). $CD8^+$ T cells were suspended at 5×10^6 cells/mL.

Coculture of Peptide-pulsed DCs and $CD8^+$ T Lymphocytes

On day 10, mature DCs were cultured with HSP-derived positive or negative peptides at a final concentration of 20 µg/mL for 2 hours in a 5% CO_2 atmosphere at 37°C. The peptide-pulsed DCs were collected and washed 3 times and suspended at 2×10^6 cells/mL. $CD8^+$ T cells at 5×10^5 cells/well and peptide-pulsed DCs at 2×10^5 cells/well were cocultured in 48-well plates in a 5% CO_2 atmosphere at 37°C. On day 12, IL-2 was added to the cultures at a final concentration of 20 U/mL.

Interferon (IFN)-γ ELISPOT Assay of Stimulated $CD8^+$ T Cells

On day 17, stimulated $CD8^+$ T cells were plated in triplicate wells at a density of 5×10^4 cells/well in the medium for IFN-γ using an ELISPOT assay, according to the manufacturer's instructions (Mabtech, Cincinnati, OH). $CD8^+$ T cells were incubated for 18–20 hours in an incubator with stimulator cells. The number of spots on each plate was determined using an Eliphoto Scan (Minerva Tech, Tokyo, Japan). Similar results were obtained in 5–26 independent experiments. This experiment has been performed at least 5 times for each peptide. Because the amount of sample was limited, the peptides that are not likely to be candidates have stopped the experiment any time. In contrast, to improve the accuracy for potential candidate peptides, additional experiments were repeated up to 26 times.

Lymphocyte Preparation for Cytotoxicity Detection Assay

PBMCs were obtained from HLA-A*24:02-positive or *02:01-positive healthy volunteers. Peripheral blood was obtained via venipuncture, collected in EDTA tubes, and transferred to the central laboratory at room temperature. Within 24 hours of blood collection, PBMCs were isolated using Ficoll-Paque (Amersham Pharmacia Biotech) density gradient solution. For in vitro culture, the PBMCs were thawed simultaneously, and 1×10^6 cells/well were incubated in medium with peptide stimulation (20 µg/mL) performed twice on days 1 and 8 in combination with the HIV-A*24:02 (RYLRDQQLL) and HIV-A*02:01 (SLYNTVATL) epitope peptides as negative controls. A total of 20 IU/µL of recombinant IL-2 (Novartis) was added on days 2, 5, 9, and 13. On day 15, the cultured lymphocytes were subjected to an xCELLigence cytotoxicity detection assay after negative selection of $CD8^+$ T cells using magnetic beads (Miltenyi Biotec). xCELLigence cytotoxicity detection assays were performed as described.

xCELLigence Cytotoxicity Detection Assay

All experiments were performed using the respective target cell culture medium. A 50-µl aliquot of the medium was added to E-Plates 16 (ACEA Biosciences, San Diego, CA) for measurement of background values. Target cells were seeded in the medium at a density of 10,000 cells/well. Suitable cell densities were determined by prior titration experiments. Cell attachment was monitored using an RTCA SP instrument (Roche) with RTCA software, version 1.1 (Roche), until the plateau phase was reached, which usually required ~22–26 hours T cells were added at different effector to target (E:T) ratios, ranging from 20:1 to 5:1. Upon addition of effector cells, impedance measurements were performed every 15 minutes for up to 81 hours. All experiments were performed in duplicate. Changes in electrical impedance were expressed as a dimensionless cell index (CI) value, which was derived from relative impedance changes corresponding to cellular coverage of the electrode sensors, normalized to baseline impedance values with medium only. To analyze the acquired data, CI values were exported and the percentage of lysis was calculated in relation to control cells lacking any effector T cells. Percent lysis was determined using the following formula at various time points: (CI tumor only−[CI tumor+T cells])/(CI tumor only)×100.[16]

Induction of HSP70-derived Peptide-specific CTLs in Vivo

HLA-A 24 transgenic (Tg) mice were kindly provided by Dr Nishimura and Dr Irie (Lemonnier, unpublished data). Mice were maintained at the Yamaguchi University Animal Facility (Ube, Japan) under specific pathogen-free conditions. All experiments used mice that were 8–12 weeks old at the time of the first procedure. All mice were used according to the guidelines of the institutional animal care and use committee of Yamaguchi University, which approved this study (ID number: 33038).

HLA-A 24 Tg mice were subcutaneously immunized with PBS, adjuvant of 50 µg poly(I:C) plus 1 µg LAG-3-Ig, or 50 µg of HSP70-derived peptide with adjuvant of 50 µg poly(I:C) plus 1 µg LAG-3-Ig[17] once a week for 8 weeks. At 7 days after the last immunization, lymph nodes were extracted and homogenized. For the in vitro culture, 5×10^5 lymphocytes/well were maintained in complete medium consisting of RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 2 mM glutamine, 100 U/mL penicillin G, and 100 g/mL streptomycin sulfate with HSP70-derived peptide (10 µg/mL) after lysing red blood cells in ACK lysis buffer. Five days later, the lymphocytes were harvested and the mouse IFN-γ ELISPOT assay (Mabtech, Nacka Strand, Sweden) was performed according to the manufacturer's protocol. Because of the small number of cells, lymphocytes from each group of mice were mixed and used in the ELISPOT assay. Briefly, lymphocytes (3×10^6 cells/80 µL) were added to a 96-well ELISPOT plate pre-coated with anti-mouse monoclonal IFN-γ antibodies. Experiments were carried out in triplicate. The cells were incubated with 10 µg/mL of HSP70-derived peptide for 24–48 hours. After stimulation, the wells were washed and incubated with biotinylated anti-mouse IFN-γ antibody for 2 hours at room temperature. Subsequently, the wells were washed and incubated with streptavidin-horseradish peroxidase for 1 hour. Spot-forming cells were developed with TMB substrate and counted using an Eliphoto Scan (Minerva Tech).

Statistical Analysis

Data are presented as mean and SD. Statistical differences between 2 groups were evaluated using the unpaired Student t test with JMP software, version 12.0 (SAS Institute Japan, Tokyo, Japan). P-values <0.05 were considered significant.

RESULTS

Analysis of Binding of HSP70-derived Peptides to HLA-A*24:02, *02:01, and *02:06

HSP70-derived 9-mer peptides that exhibited high binding affinity to HLA-A*24:02, 02:01, and 02:06 were selected as candidate peptides using a binding prediction system (NEC Corporation) that utilizes a data-mining technique and a query learning algorithm based on hidden Markov models.[7]

For this study, we examined binding to HLA-A*24:02, *02:01, and *02:06. HLA-A*24:02 is the most frequent allele among Asians (eg, 33% in Japanese[18] versus ~10% in western populations). Seventeen peptides exhibiting an affinity of 4.0 or higher in terms of log Ka for binding to at least 1 of the 3 HLA-A types were selected as targets for the experiment (Table 1). To achieve therapeutic effects in a wide range of cases, peptides with high affinity for all 3 HLA-A molecules would be more preferable, and these are indicated in bold font in Table 1.

Production of IFN-γ by CTL Clones Induced by Candidate Peptides

HLA-A*24:02-positive, *02:01-positive, and *02:06-positive CD8$^+$ T cells were pulsed with respective peptides, and then IFN-γ production was analyzed by ELISPOT assay. Data for positive and negative controls are significantly different. Four peptides induced peptide-specific CTLs producing IFN-γ against HLA-A*24:02-positive CD8$^+$ T cells, and 2 peptides induced peptide-specific CTLs producing IFN-γ targeting HLA-A*02:01-positive and *02:06-positive CD8$^+$ T cells (Fig. 1). Seven of the HLA-A*24:02-positive patients (represented by black bars) had HLA-A*02:01 or HLA-A*02:06. Compared with the negative control, we obtained both HLA-A*24:02-positive CTLs in addition to HLA-A*02:01-positive and *02:06-positive CTLs that specifically produced significant amounts of IFN-γ after CTL expansion when pulsed with peptide Y50. Although HLA-A*24:02-positive Y75 peptide-specific CTLs produced a significant amount IFN-γ as compared with the negative control, HLA-A*02:01-positive and *02:06-positive Y75 peptide-specific CTLs did not. Compared with the negative control, CTLs induced with other peptides did not exhibit significant IFN-γ production, regardless of HLA-A alleles.

Cytotoxic Activity of CTLs

We also examined the cytotoxic activity of CTL clones to evaluate the possibility of developing a cancer vaccine for use in tumor therapy involving candidate peptides. We wished to identify a peptide that could induce all HLA-A*24:02-positive CTLs and HLA-A*02:01-positive and *02:06-positive CTLs promiscuously. Therefore, we used the Y50 peptide for this experiment. Other peptides could not induce CTLs to a similar degree as the Y50 peptide. Therefore, we did not expect cytotoxicity to other peptides and did not conduct experiments using these peptides. A peptide Y50-specific CTL clone with HLA-A*24:02 and *02:01 demonstrated cytotoxic activity against HLA-A*24:02-positive and *02:01-positive HepG2 cells, which express high levels of HSP70. In contrast, HIV peptide–specific CTL clones did not demonstrate cytotoxicity against HepG2 cells (Fig. 2).

Induction of Specific CTLs by Vaccination with HSP70-derived Peptides in Mice

We next examined whether peptide Y50 induces HSP70-specific CTLs in HLA-A 24 Tg mice. Following 8 subcutaneous vaccinations with PBS, adjuvant of (I:C) plus LAG-3-Ig, or peptide Y50 with adjuvant of poly(I:C) plus LAG-3-Ig, lymphocytes were cultured in vitro and then
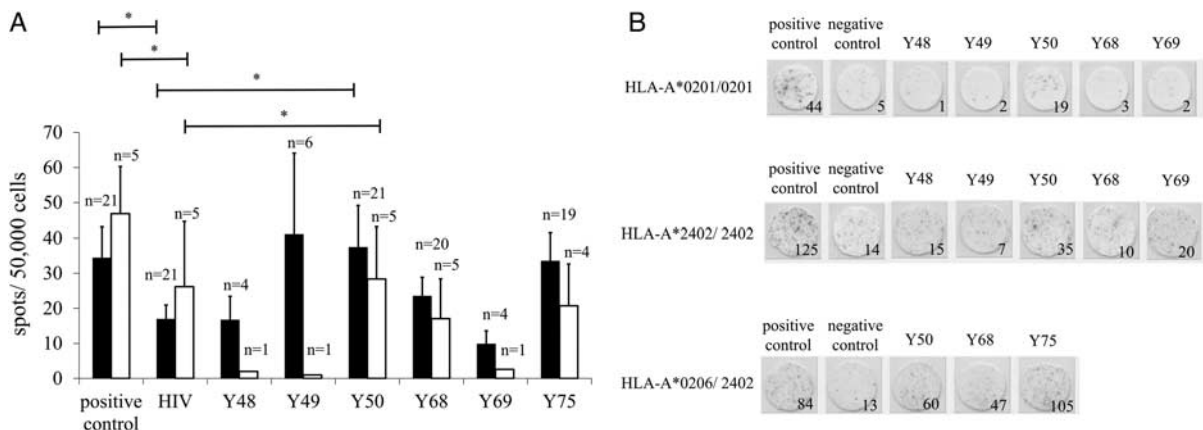

FIGURE 1. IFN-γ production by CTLs induced by heat-shock protein 70-derived peptides. A, IFN-γ production by CTLs from HCC patients induced by self-DCs pulsed with heat-shock protein 70-derived peptides. IFN-γ production was examined using an IFN-γ ELISPOT assay. Depending on the status of the allele, the HLA-A*24:02-restricted EBV peptide (TYGPVFMCL), HLA-A*02:01-restricted Flu A peptide (GILGFVFTL), and HLA-A*02:06-restricted EBV peptide (LTAGFLIFL) were used as positive controls. The HLA-A*24:02-restricted HIV (RYLRDQQLL), HLA-A*02:01-restricted HIV (SLYNTVATL), and HLA-A*02:06-restricted HIV (ATLEEMMTA) peptides were used as negative controls in the same manner. "Closed bars" indicate mean IFN-γ production by CTLs from HLA-A*24:02-positive HCC patients responding to self-DCs pulsed with indicated peptide, and "open bars" indicate mean IFN-γ production by CTLs from HLA-A*24:02-negative and HLA-A*02:01-positive or *02:06-positive HCC patients responding to self-DCs pulsed with indicated peptide. All experiments were performed in triplicate at a density of 5×10^4 cells/well. This experiment was performed at least 5 times in total for each peptide. Because the amount of sample was limited, experiments were not repeated beyond 5 times for peptides deemed unlikely candidates. However, to improve the accuracy for potential candidate peptides, additional experiments were repeated up to 26 times. Data represent the mean ± SD of the IFN-γ release. *P < 0.05. B, The representative data are shown. Peptide Y50-specific spots were increased in patients with HLA-A*24:02, *02:01, or *02:06. CTL indicates cytotoxic T lymphocyte; DC, dendritic cell; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; IFN, interferon.
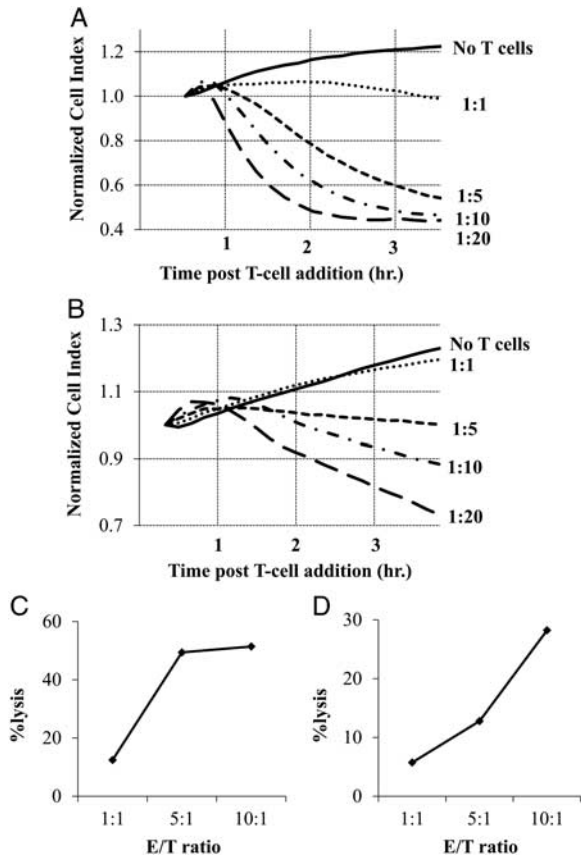
FIGURE 2. Impedance traces of HepG2 tumor cells incubated alone or with varying concentrations of HLA-A*24:02-positive (A) or *02:01-positive (B) tumor-specific T cells. Each trace was calculated from triplicate data points collected every 5 minutes through the duration of the culture. C and D, Data for percent lysis of HepG2 cells in response to varying concentrations of heat-shock protein 70–derived peptide-specific T cells measured 5 hours after mixing of tumor and T cells, as determined using an impedance-based assay. C and D, indicate calculated data of (A) and (B), respectively. Percent lysis was determined using the following formula: (CI tumor only–[CI tumor+T cells])/(CI tumor only)×100. CI indicates cell index; E/T, effector to target.

evaluated for IFN-γ production. Lymphocytes from 3 mice immunized with peptide Y50 exhibited significantly higher IFN-γ production (Fig. 3).

We also examined the tissues of the 3 immunized mice that exhibited HSP70 peptide–specific CTL responses. In all 3 mice, no pathologic changes caused by immune responses, such as lymphocyte infiltration or tissue destruction and repair, were noted in the brain or other internal organs except for the intestines, and the tissues exhibited normal structures and cellularity. Minimal to mild cellular infiltration was observed in the intestines of mice in the adjuvant group and adjuvant plus peptide group (Table 2).

DISCUSSION

In this study, we performed binding assays to identify candidate HSP70-derived peptides and identified a multi-HLA-class I–binding epitope peptide that binds to HLA-A*02:01, *02:06, and *24:02 by immune induction using an in vitro IFN-γ ELISPOT assay. We confirmed cytotoxicity
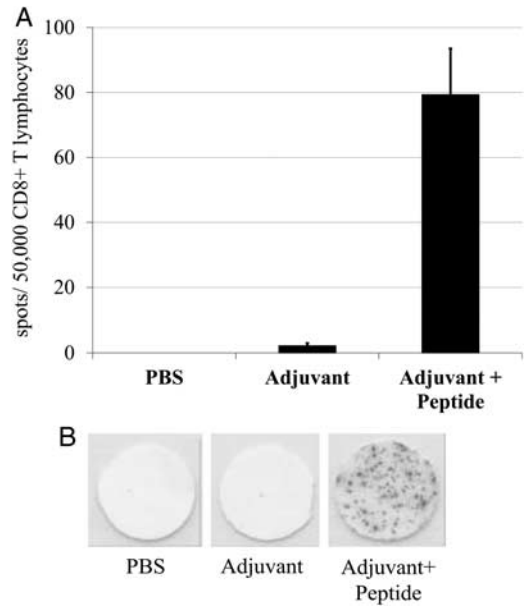

FIGURE 3. A, HLA-A24 Tg mice were immunized with phosphate buffered saline, adjuvant of 50 μg poly(I:C) plus 1 μg LAG-3-Ig, or 50 μg of heat-shock protein 70-derived peptide with adjuvant of 50 μg poly(I:C) plus 1 μg LAG-3-Ig once a week for 8 weeks. At 7 days after the last immunization, lymph nodes were harvested. Lymphocytes were cultured for 5 days with heat-shock protein 70-derived peptide (10 μg/mL) and then subjected to interferon-γ ELISPOT assay. Because of the small number of cells, lymphocytes from each group of mice were mixed and used in the ELISPOT assay. Experiments were carried out in triplicate. Data are reported as mean ± SEM for each group. Lymphocytes of 3 mice immunized with peptide Y50 exhibited significantly greater interferon-γ production than those of the other groups. B, Representative data are shown. PBS indicates phosphate buffered saline.

in vitro and induction of an immune response and safety in vivo using HLA-A 24 Tg mice. In this study, we targeted HLA-class I peptides capable of binding to multiple HLAs. We used a peptide prediction system (developed by NEC Corporation) appropriate for screening promiscuous peptides identified in previous reports as capable of binding to HLA-class I molecules.

Peptide binding assays were performed for candidates selected from the binding prediction system results, and the ability of these peptides to induce an immune response was verified using the IFN-γ ELISPOT assay. Peptide Y50 did not exhibit the highest binding affinity for each HLA, but it did induce immune responses against multiple HLAs. As HSP70 is a relatively ubiquitous molecule, peptides derived from HSP70 could be considered cryptic (ie, immune tolerance could result if the binding affinity of the peptide was too high).[19] Regarding HLA-A*02:06, CTL induction was confirmed in the patients with HLA-A*02:06 (Supplemental Fig., Supplemental Digital Content 1, http://links.lww.com/JIT/A530). However, the frequency of HLA-A*02:06 in the Japanese population is reportedly ~9%, and only 2 patients in our study had HLA-A*02:06. In addition, cases with HLA-A*02:06 always had HLA-A*24:02 or *02:01. Thus, unfortunately, we could not determine whether this CTL induction was against HLA-A*02:06 or another allele. However, for the A2 supertype, some reports have indicated

TABLE 2. Histopathologic Findings of Immunized Mice

	PBS (n = 1)	Adjuvant (n = 2)	Ajuvant+Peptide (n = 3)
Cerebrum	Not remarkable	Not remarkable	Not remarkable
Heart	Not remarkable	Not remarkable	Not remarkable
Lung	Not remarkable	Not remarkable	Not remarkable
Liver	Not remarkable	Not remarkable	Not remarkable
Pancreas	Not remarkable	Not remarkable	Not remarkable
Kidney	Not remarkable	Not remarkable	Not remarkable
Intestine	Not remarkable	Cell infiltration: minimal–mild	Cell infiltration: minimal–mild
Ovary	Not remarkable	Not remarkable	Not remarkable

PBS indicates phosphate buffered saline.

that the peptide for HLA-A*02:01 exhibits cross-reactivity to *02:06 when the binding affinity is high and therefore induces CTLs.[20,21] In this study, the Y50 peptide exhibited high binding affinity to HLA-A*02:01, and CTL induction was also observed, suggesting cross-reactivity to HLA-A*02:06. We hope to collect samples and verify this in future experiments.

With respect to cytotoxicity, a peptide Y50-specific CTL clone with HLA-A*24:02 and *02:01 exhibited cytotoxicity against HSP70-expressing cells (HepG2), and this result was consistent with results obtained from the binding and immune response induction assays. Cytotoxicity was evaluated based on electrical impedance measurements using the xCELLigence System. It is reported that a small number of specific T cells and a low effector to target cell ratio commonly leads to a delay in maximal lysis well beyond 24 hours. Using conventional techniques to determine the cytotoxic potential of antigen-specific T cells, such as the ^{51}Cr-release assay, it is not possible to draw conclusions regarding the lysis kinetics of T cells because these are typically endpoint assays. In contrast, xCELLigence is a convenient label-free method that is particularly applicable to the detection of antigen-specific T-cell–mediated cytotoxicity by monitoring lysis over time.[22] In this study, we confirmed that cytotoxicity increased with time.

In vivo experiments were performed by administering poly(I:C) plus LAG-3-Ig in combination with peptide to HLA-A 24 Tg mice. Although the efficacy of peptide vaccine anticancer therapies administered alone has generally been limited, recent research indicated that the therapeutic effect of peptide drugs can be enhanced by combining their administration with adjuvants such as immune checkpoint inhibitors.[2,23–25] The combination of poly(I:C) and LAG-3-Ig were shown to improve the antitumor effects of cancer vaccines by preventing T-cell exhaustion, and these agents are currently available in clinical practice.[17] For this reason, we used combined adjuvants in our in vivo experiments and noted sufficient immune response induction, with no severe adverse events observed. However, we could not verify immune checkpoint changes in the present study due to the lack of an adequate number of samples. In addition, in vivo verification of HLA-A 02 was not carried out because we could not obtain HLA-A 02 Tg mice. These issues will be addressed in future studies.

The present results demonstrate that irrespective of HLA-A*24:02, *02:01, or *02:06 status, DCs pulsed with a specific HSP70-derived peptide induced specific CTLs to exhibit antitumor activity. The immunogenicity of the HSP70-derived peptide should be examined in patients with HSP70-expressing cancers, and we are currently conducting appropriate clinical trials.

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