Targeting cryptic epitope with modified antigen coupled to the surface of liposomes induces strong antitumor CD8 T-cell immune responses *in vivo*

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Abstract. Active cancer immunotherapy, such as cancer vaccine, is based on the fundamental knowledge that tumor-associated antigens (TAAs) are presented on MHC molecules for recognition by specific T cells. However, most TAAs are self-antigens and are also expressed on normal tissues, including the thymus. This fact raises the issue of the tolerance of the TAA-specific T-cell repertoire and consequently the inability to trigger a strong and efficient antitumor immune response. In the present study, we used antigens chemically coupled to the surface of liposomes to target telomerase reverse transcriptase (TERT), a widely expressed self/tumor antigen. Taking advantage of the high homology between mouse and human TERT, we investigated immunogenicity and antitumor efficiency of the liposomal TERT peptides in HLA-A*0201 transgenic HHD mice. Using the heteroclitical peptide-modifying approach with antigen-coupled liposomes, we identified a novel cryptic epitope with low affinity for HLA*0201 molecules derived from TERT. The heteroclitical variant derived from this novel low affinity peptide exhibited strong affinity for HLA*0201 molecules. However, it induced only weak CD8 T-cell immune responses in HHD mice when emulsified in IFA. By contrast, when coupled to the surface of the liposomes, it induced powerful CD8 T-cell immune responses which cross-reacted against the original cryptic epitope. The induced CD8 T cells also recognized endogenously TERT-expressing tumor cells and inhibited their growth in HHD mice. These data suggest that heteroclitical antigen derived from low affinity epitope of

tumor antigens coupled to the surface of liposome may have a role as an effective cancer vaccine candidate.

Introduction

Tumor-associated antigen (TAAs)-specific T cells are detected in many patients with cancer (1,2). However, these TAA-specific T cells typically fail to control tumor growth. It is expected that vaccination with TAA enhances tumor-specific T cell responses, improves antitumor immunity and provides clinical benefit. The development of cancer vaccines has been greatly potentiated by the availability of synthetic tumor-antigen-peptides, although progress has been hampered by the poor immunogenicity of antigen-peptides.

Liposome has been proposed to be a carrier of antigens and an adjuvant to induce immune responses (3). We previously reported that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acids were cross-presented by antigen-presenting cells (APCs) to cytotoxic T lymphocytes (CTLs) (4). Moreover, we have shown that single immunization of as low as 280 ng of liposomal peptide along with CpG conferred complete protection to the immunized mice from a highly virulent mutant strain, clone 13 of lymphocytic choriomeningitis virus (LCMV). In addition, surface-linked liposomal antigens induced long-lived memory CD8$^+$ T cells in the absence of CD4$^+$ T-cell assistance (5,6). This form of CTL-based liposomal vaccine was also investigated for tumor vaccine using an OVA-expressing mouse tumor model. This investigation suggested surface-coupled liposomal antigens may be applicable for the development of tumor vaccines (4). However, to the best of our knowledge, the possibility of a vaccine targeting endogenous tumor antigen by surface-coupled liposomal antigens remains to be examined.

Telomerase reverse transcriptase (TERT) is a tumor antigen that is highly expressed in >85% of all human types of cancer including stem cell-like tumor cells (7-10). Therefore, TERT antigens have the potential to mediate antitumor immune responses against a range of tumors (11). In previous studies, epitope peptides derived from TERT have been shown to elicit CTL responses capable of recognizing TERT-expressing tumor cells *in vitro* (7,8,12). However, reports of antitumor efficiency *in vivo* using natural TERT peptide are limited.

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Self-antigen peptides do not necessarily undergo antigen processing/presentation and serve as dominant epitopes. In certain situations, the peptides are hidden from immune recognition and termed 'cryptic' (13). Dominant peptides show a high affinity for MHC molecules and are frequently presented by somatic cells, whereas, cryptic peptides show a low affinity for MHC molecules and are infrequently presented by somatic cells. Similar to many other TAAs, TERT is a self-antigen. Therefore, it is thought that central tolerance against TERT-derived peptides with high affinity for MHC molecules exists. The central tolerance against TAAs is deemed to be a major barrier for TAA-specific antitumor immunotherapy. On the other hand, the CTL repertoire specific for cryptic epitope may escape from the negative selection process in central tolerance. Therefore, CTLs against cryptic epitopes may be available for antitumor immunotherapy. However, it is difficult to identify cryptic epitopes for immunotherapy due to their poor immunogenicity. To circumvent this problem, previous investigators have created modified heteroclitical peptides to enhance their affinity for HLA-A2 molecules while preserving the amino acids that interact with the T-cell receptor (TCR), which is specific to the original cryptic epitope (14-17). Previous findings showed that vaccination with heteroclitical peptide derived from cryptic epitopes of TERT inhibited the growth of tumors *in vivo* (15,23).

The aim of the present study was to determine the possibility that the liposome coupled with a peptide derived from TERT may serve as an effective CTL-based vaccine against tumor. First, to identify the optimum peptide for coupling on the surface of liposomes, 20 TERT-derived peptides with HLA-A2.1 binding motif were selected by computer algorism, the liposome-coupled peptide was prepared and their ability to provoke peptide specific CD8 T cell responses in HLA-A*0201-transgenic (HHD) mice was determined. We identified a novel cryptic epitope with low affinity for HLA molecules and found that immunization using liposome-coupled heteroclitical peptide derived from this novel cryptic epitope elicits CD8 T-cell responses against native peptide and efficiently inhibits the growth of TERT expressing tumors in HHD mice. Our results strongly support the use of liposomal vaccine surface coupled with TERT antigens as a new potential option for active immunotherapy against tumors.

Materials and methods

Prediction of CTL epitopes. To define potential HLA-A*0201-binding peptides from human TERT (hTERT), a computer-based program, BIMAS (http:// www-bimas.cit. nih.gov/molbio/hla_bind/), was used. As shown by the odd numbers in Table I, 10 peptides that have superior Bimas scores and homologous sequence in murine TERT (mTERT) were selected for predicted CTL epitopes.

Modified heteroclitical peptides were produced to enhance their affinity for HLA-A2 molecules while preserving the amino acids that interact with the TCR as previously described (18-21). Heteroclitical modifications were performed by replacing the amino acid at position 1 with a tyrosine (Y), position 2 with a leucine (L) or position 9 with a valine (V). Heteroclitical peptides are shown by the even numbers in Table I. These peptides were synthesized by Eurofins Genomics (Tokyo, Japan).

Animals. The HLA-A*0201-transgenic HHD mice (a kind gift from Dr F.A. Lemonnier, Pasteur Institute, Paris, France) expressed a transgenic HLA-A*0201 monochain in which human β-2 microglobulin (β2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α1 and α2, domains) and H-2Db (α3, transmembrane and cytoplasmic domains) (18). Six- to 12-week-old mice were used for all the experiments. The mice were housed in appropriate animal care facilities at Saitama Medical University (Saitama, Japan) and handled according to international guidelines. Experimental protocols were approved by the Animal Research Committee of Saitama Medical University.

Measurement of peptide relative affinity (RA) for HLA-A2.1. T2 cells ($3x10^5$/ml) were incubated with various concentrations of peptides ranging from 100 to 0.1 μM in serum-free RPMI-1640 medium (R0) supplemented with 100 ng/ml human β2m (Acris Antibodies, San Diego, CA, USA) at 37°C for 16 h, as previously described (16). The cells were then stained with the HLA-A2.1 molecule-specific BB7.2 mAb (BioLegend, San Diego, CA, USA). For each peptide concentration, HLA-A2.1-specific staining was calculated as the percentage of the staining obtained with 100 μM of the reference peptide HIVpol589 (IVGAETFYV). The RA was determined as: RA = concentration of each peptide that induces 20% of HLA-A2.1 expression/concentration of the reference peptide that induces 20% of HLA-A2.1 expression. Lower RA value shows higher binding affinity for HLA-A2.1.

Assessment of peptide/HLA-A2.1 complex stability. T2 cells were incubated with 100 μM of each peptide in R0 supplemented with 100 ng/ml human β2m at 37°C for 16 h, as previously described (16). The cells were then washed four times to remove free peptides, incubated with 0.2% GoldiPlug (BD Biosciences Pharmingen, San Diego, CA, USA) in R0 for 1 h to block the cell surface expression of newly synthesized HLA-A2.1. After washing and incubation for 0, 2, 4, 6 or 8 h, the cells were stained with anti-HLA-A2.1 antibody (BB7.2). For each time-point, the peptide-induced HLA-A2.1 expression was calculated as: mean fluorescence of peptide-preincubated T2 cells - mean fluorescence of T2 cells treated in similar conditions in the absence of peptide. DC_{50} (dissociation complex, DC) was defined as the time required for the loss of 50% of the HLA-A2.1/peptide complexes stabilized at t=0.

Cell lines and telomerase detection. Mouse lymphoma cell lines RMA and RMA-HHD, which were a kind gift from Dr F.A. Lemonnier (22) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (R-10). Telomerase detection in tumor cells was achieved by flow cytometry as previously reported using anti-hTERT monoclonal antibody (H-231; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), which cross reacts with mTERT (23).

Liposome. The liposomes used in the present study were provided by NOF Corporation (Tokyo, Japan), and consisted of dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylethanolamine (DOPE), dioleoyl phosphatidyl glycerol (DOPG) and cholesterol in a 4:3:2:7 molar ratio. The crude

Table I. Bimas score, HLA-A2.1 affinity and stabilization capacity of hTERT peptides.

Peptide no.[a]	Start position	Subsequence[b]	BIMAS score[c]	RA[d]	DC$_{50}$ (h)[e]
1	540	ILAKFLHWL	1745.714	2	8
2		ILAKFLHW<u>V</u>	5683.72	2	>8
3	572	RLFFYRKSV	257.342	32	<2
4		<u>Y</u>LFFYRKSV	1183.775	4	4
5	943	LLQAYRFHA	48.984	8	4
6		LLQAYRFH<u>V</u>	685.783	4	>8
7	926	LQVNSLQTV	27.573	4	>8
8		L<u>L</u>VNSLQTV	271.948	2	>8
9	579	SVWSKLQSI	17.427	32	2
10		S<u>L</u>WSKLQSI	199.162	8	>8
11	945	QAYRFHACV	11.747	8	4
12		Q<u>L</u>YRFHACV	845.752	4	6
13	944	LQAYRFHAC	1.627	32	4
14		LQAYRFHA<u>V</u>	22.777	2	>8
15	23	PLATFVRRL	0.822	>32	4
16		<u>Y</u>LATFVRRL	171.771	4	>8
17	847	GDMENKLFA	0.576	>32	<2
18		G<u>L</u>MENKLFA	414.505	>32	>8
19	925	DLQVNSLQT	0.559	>32	<2
20		<u>Y</u>LQVNSLQT	34.279	>32	<2

[a]Odd numbers, native peptides; even numbers, heteroclitic peptides. [b]Underlines show amino acids replaced from native peptide. [c]BIMAS score was used for prediction of HLA-A*0201-binding peptides. [d]RA was determined as described in Materials and methods. Lower RA value shows higher binding affinity for HLA-A2.1. [e]DC$_{50}$ was defined as the time required for the loss of 50% of the HLA-A2.1/peptide complexes stabilized at t=0. RA, relative affinity.

liposome solution was passed through a membrane filter with a pore size of 0.2 mm.

Coupling of peptide to liposomes. Liposomal conjugates with TERT peptides were prepared essentially in the same manner as previously described (6). The final preparations contained ≤0.7 mg of the peptide and 10 mg of the liposome/ml (24).

Immunization. The mice were immunized with indicated doses of liposomal peptides via subcutaneous injection in the presence of 5 μg/mouse CpG or 100 μg of peptide emulsified in 250 μl of IFA with 100 μg of peptide HBV core 128-140. These immunizations were performed on day 0 and 7.

Intracellular IFN-γ and CD107a degranulation analysis. After 5 days following immunization, spleen cells of immunized mice were incubated with brefeldin A (GolgiPlug; BD Biosciences) and fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to CD107a for 5 h at 37˚C in the presence or absence of a relevant peptide. After the incubation, the cells were washed, labeled with peridinin chlorophyll protein complex (PerCP)-conjugated anti-CD8α mAb, fixed and permeabilized (Cytofix-Cytoperm; BD Biosciences) before they were labeled with phycoerythrin (PE)-conjugated anti-IFN-γ mAb. All the antibodies were purchased from Biolegend. The stained cells were analyzed on a FACSCant II flow cytometer (BD Biosciences).

HLA-A2.1 dextramer analysis. To quantify antigen-specific T cells, the spleen cells were incubated with allophyco-cyanin (APC)-conjugated HLA dextramer recognizing 944v peptide derived from hTERT (944v/dextramer; Immudex, Copenhagen, Denmark) for 20 min at room temperature. After the incubation, the cells were washed, labeled with PerCP-conjugated anti-CD8α mAb. The stained cells were analyzed on a FACSCant II flow cytometer (BD Biosciences).

CTL generation and cytotoxic assay. After 5 days following immunization, the spleen cells of immunized mice were cultured for 6 days with irradiated (30 Gy), syngeneic naïve spleen cells pre-pulsed with a relevant peptide and employed as effector cells in CTL-mediated cytotoxic assay. TERT-positive RMA-HHD cells were used as target cells. CTL-mediated cytotoxic assay was performed using a LIVE/DEAD Cell-mediated Cytotoxicity Assay kit (Life Technologies, Carlsbad, CA, USA) according to the manufac-turer's instructions.

In vivo tumor protection. The HHD mice were vaccinated with liposomal peptide with 5 μg of CpG on day 14, 7 and 0. On day 0, the mice were challenged subcutaneously with 2x10⁴ RMA-HHD cells. Tumor size was monitored every 3 to 4 days using calipers and the mice were sacrificed when the tumor mass reached >1,000 mm² or tumor tissue disintegration occurred.

Table II. Contents of peptide groups.

Peptide groups	Peptide no.
1	1, 5, 9, 13, 17
2	3, 7, 11, 15, 19
3	2, 6, 10, 14, 18
4	4, 8, 12, 16, 20

Statistical analysis. Data were presented as the mean ± SD. Statistical comparison between groups was based on the Mann-Whitney U test using Microsoft Excel. Mouse survival time was estimated using the Kaplan-Meier method and the Log-rank test. P<0.05 was considered significant.

Results

Selection of CTL epitopes derived from hTERT for coupling to liposomes. Ten native peptides derived from hTERT were selected by the BIMAS score (odd numbers in Table I). To improve the binding affinity for HLA-A2.1, these peptides were subjected to amino acid substitutions as shown in Table I (even numbers). Most of the heteroclitical peptides improved their RA and DC_{50} value compared with the native peptides (Table I). To assess the immunogenicity, these peptides were pooled into four groups consisting of five peptides (Table II) and coupled to the surface of liposomes.

Figure 1. Immunogenicity of predicted liposomal peptides. Spleen cells from HHD mice vaccinated with a peptide pool conjugated to liposomes were restimulated *in vitro* with a relevant peptide for 5 h. CD8 T cells were tested for reactivity to each peptide by flow cytometric assay using the degranulation of CD107a and intracellular IFN-γ staining. Percentages of IFN-γ+ CD107a+ cells in CD8+ spleen cells are presented as the mean ± SD of three mice.
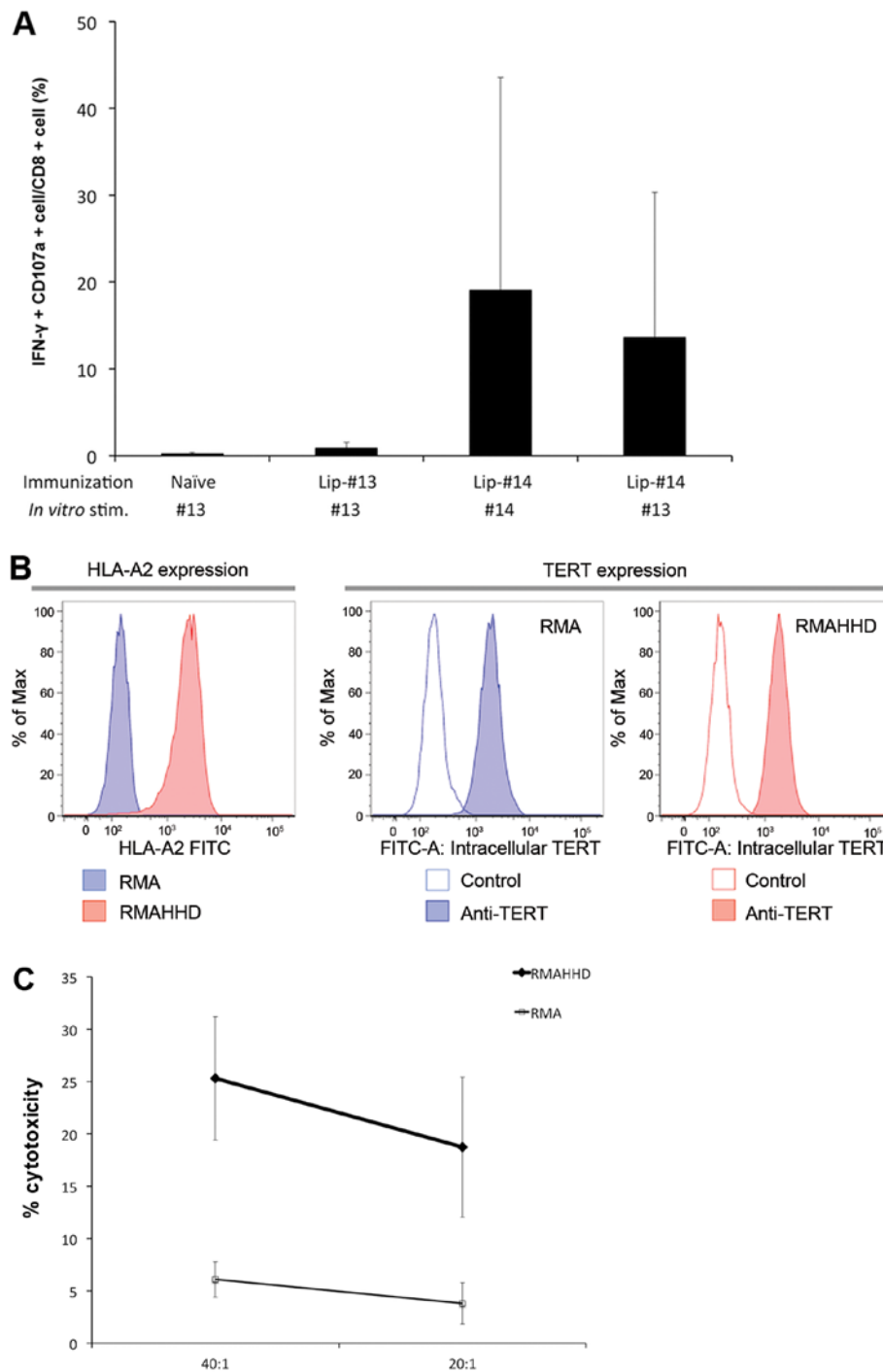
Figure 2. Generation of native cryptic epitope reactive CD8 T cells by vaccination with liposomal heteroclitic valiant. (A) CD8 T cells from Lip-#14 immunized mice recognized #14 as well as native epitope #13. Spleen cells from the HHD mice vaccinated with Lip-#13 (native) or Lip-#14 (heteroclitic) were restimulated *in vitro* with #13 or #14 peptide for 5 h. CD8 T cells were tested for specificity of each peptide by flow cytometric assay using the degranulation of CD107a and intracellular IFN-γ staining. Results are presented as the mean ± SD of three mice. (B) Flow cytometric analysis of HLA-2.1 and TERT expression on RMA and RMA-HHD mouse lymphoma cell lines. (C) CTL activities against TERT-expressing tumor cells. Spleen cells from the HHD mice vaccinated with Lip-#14 were restimulated *in vitro* with #14 peptide. After 6 days, the CTL-mediated cytotoxic assay was performed with TERT-expressing RMA and RMA-HHD cells as the target.

Each liposomal peptide pool was subsequently injected into the HHD mice. After 5 days following immunization, the spleen cells of immunized mice were prepared, stimulated with a relevant peptide and stained for their expression of surface CD8 and intracellular IFN-γ and degranulation of CD107a. As shown in Fig. 1, three heteroclitical peptides on the surface of liposomes (Lip-#8, -#10 and-#14) were able to induce a significant amount of IFN-γ⁺ CD107a⁺ CD8⁺T cells and the responses were much higher compared to those induced by the original peptides (Lip-#7, -#9 and-#13). Lip-#14 was most effective for the induction of peptide-specific IFN-γ⁺CD107a⁺ CD8⁺ T cells. Thus, #14 (p944v), the hetero-clitical peptide derived from #13 (p944), was selected as a candidate epitope.
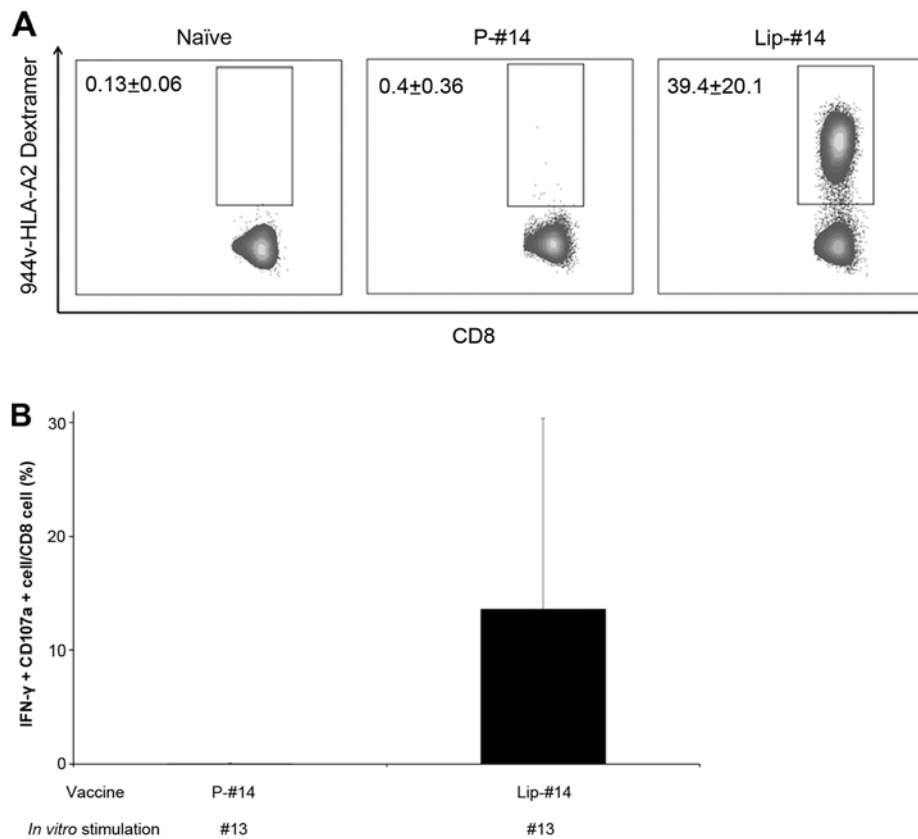
Figure 3. Comparison of native cryptic epitope specific CD8 T-cell responses in mice immunized with #14 peptide conjugated liposomes or with #14 peptide emulsified in IFA. (A) Spleen cells from naïve HHD or HHD mice vaccinated with Lip-#14 or with P-#14 were stained with #14/HLA-A2.1-dextramer. Representative FACS plot of dextramer staining and the mean ± SD of dextramer⁺ CD8⁺ cells of three mice are presented. (B) Spleen cells from HHD mice vaccinated with Lip-#14 or with P-#14 were restimulated *in vitro* with #13 peptide for 5 h. CD8 T cells were tested for specificity of the peptide by flow cytometric assay using the degranulation of CD107a and intracellular IFN-γ staining.

The heteroclitical peptide coupled to liposome (Lip-#14) induces a strong CD8 T-cell response with cross recognition to the native epitope and *in vivo* primed CTLs with Lip-#14 recognizes endogenously mTERT-expressing tumor cells.

The #13 peptide (p944) and #14 peptide (p944v) were, respectively, coupled to the surface of liposomes (Lip-#13 and -#14). These liposomal peptides were subsequently injected into the HHD mice and an intracellular IFN-γ and CD107a degranulation analysis was conducted to assess antigen-specific CD8 T-cell responses against the native epitope #13 (p944). As shown in Fig. 2A, Lip-#14 induced strong CD8 T-cell responses *in vivo*. Compared with immunization with liposomal native #13 peptide, the magnitude of CD8 responses induced by liposomal #14 peptide was extremely strong. The results also showed that CD8 T cells that derived from Lip-#14-immunized mice crossreacted with the native epitope #13 equally to the heteroclitic epitope #14.

Additionally, to evaluate whether #13 peptide is naturally processed and presented by tumor cells, #14-specific CTLs were tested for their ability to lyse endogenously mTERT-expressing HLA-A2.1⁺ tumor cells. CTLs specific for #14 were generated from the spleen cells of mice immunized with Lip-#14 and their cytolytic activity was tested against RMA-HHD mouse lymphoma cells. Fig. 2B shows that RMA-HHD cells express mTERT endogenously while also possessing sufficient cell surface HLA-A2.1. We found that CTLs specific for #14 were able to lyse RMA-HHD cells, but no cytotoxicity was observed

against HLA-A2.1-negative RMA cells (Fig. 2C). These results indicated that #13, the low affinity cryptic epitope, is naturally processed and presented by HLA-A2.1 molecules on the surface of TERT-expressing tumor cells and CTLs primed *in vivo* with Lip-#14 immunization are able to recognize and kill tumor cells in an HLA-A2.1-restricted manner.

Liposomal #14 peptide induces a stronger antigen-specific CD8 T-cell response than peptide + IFA immunization. Two vaccines were produced: Lip-#14 and a #14 peptide emulsified in IFA with a MHC class II pan T-helper peptide, HBVcore128-140 (24) (P-#14). The immunogenicity of these two forms of TERT 944v was compared by analyzing spleen cells from the immunized mice using a TERT 944v-HLA-A2 dextramer assay (Fig. 3A), intracellular IFN-γ and CD107a degranulation analysis (Fig. 3B). We found that an immunization with Lip-#14 induced greater antigen-specific CD8 T cell response compared with the P-#14 vaccination. The frequencies of TERT 944v-HLA-A2 dextramer⁺ CD8 T cells detected in HHD mice were ~100-fold higher after Lip-#14 vaccination, compared to P-#14 vaccination (39.4±20.1 vs. 0.4±0.36%). As dextramer analysis did not discriminate between anergic and functional T cells, we also monitored an antigen-specific response by intracellular IFN-γ and CD107a degranulation analysis. In agreement with results from the dextramer analysis, the frequencies of intracellular IFN-γ and CD107a⁺ CD8 T cells in response to native the #13 epitope detected
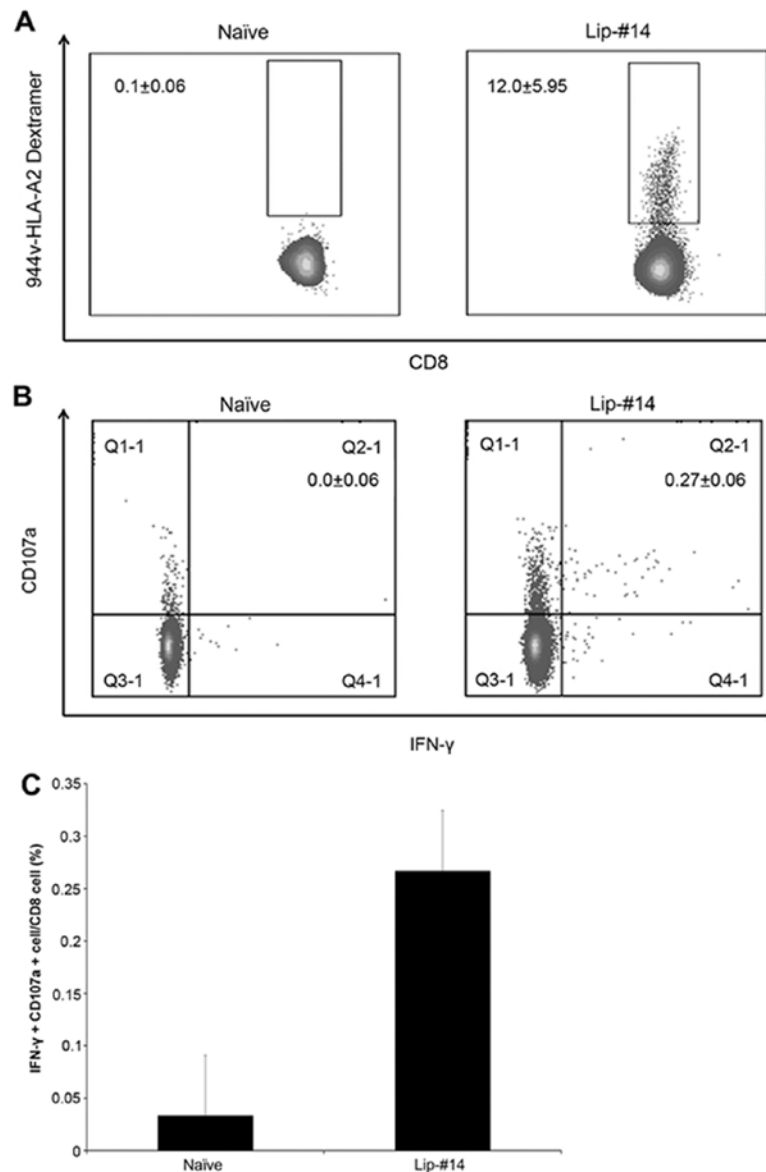
Figure 4. Induction of long-lasting memory CD8 T cells. (A) Presence of long-lasting antigen-specific CD8 T cells. HHD Mice were immunized with Lip-#14. On day 35 after immunization, spleen cells were stained with #14/HLA-A2.1-dextramer. Representative FACS plot of dextramer staining and the mean± SD of dextramer⁺ CD8⁺ cells of three mice are presented. (B and C) Immune responses of long-lasting antigen-specific CD8 T cells. The abovementioned spleen cells were restimulated *in vitro* with #13 peptide for 5 h. The CD8 T cells were tested for specificity of the peptide by flow cytometric assay using the degranulation of CD107a and intracellular IFN-γ staining.

in Lip-#14-vaccined HHD mice were in contrast with the P-#14-vaccined group (Fig. 3B). These results demonstrated that vaccination with surface-coupled liposomal antigen, which was modified to enhance their affinity for class I molecules causes strong immune responses against an epitope that cannot induce immune responses by ordinary vaccination such as peptide with IFA.

Previous reports suggest that the efficacy of active immunotherapy may be associated with the capacity of the vaccine to promote sustained CD8 T-cell responses *in vivo* (25). Therefore, we investigated whether long-lasting #14-specific and #13-crossreactive CD8 T cells could be elicited in HHD mice immunized with Lip-#14. After 35 days following immunization, the CD8 responses were measured using a dextramer assay and flow cytometric analysis using intracellular IFN-γ and CD107a degranulation. As shown in

Fig. 4A, TERT #14-specific CD8 T cells were detected in mice on day 35 after immunization with Lip-#14. When the long-lasting CD8 T-cell responses were evaluated by intracellular IFN-γ and CD107a degranulation assay, we found that significant IFN-γ⁺CD107a⁺CD8 T cells in response to native #13 peptide can only be detected in HHD mice injected with Lip-#14 (Fig. 4B and C). These results suggested that immunization with Lip-#14 is capable of effectively generating long-lasting memory CD8 T cell and sustained CD8 T-cell responses against native cryptic epitope.

Immunization with Lip-#14 induces tumor growth inhibition. To investigate the antitumor effect of Lip-#14, we immunized HHD mice with Lip-#13 or -#14 on day 14, 7 and 0. HHD mice immunized with irrelevant hepatitis C virus core 132-140 peptide coupled to the surface of liposomes were
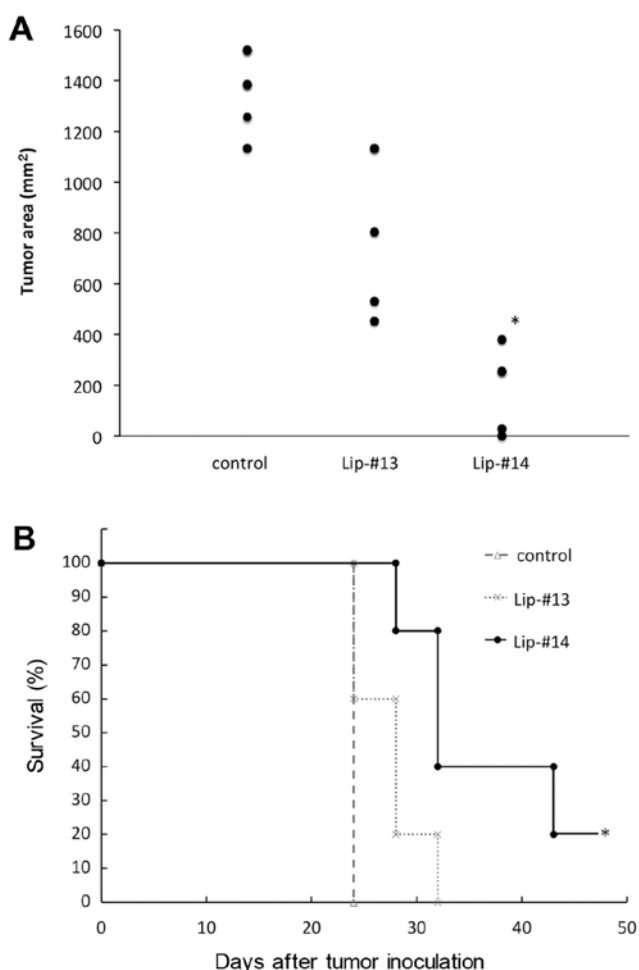
Figure 5. Immunization with liposomal heteroclitic variant inhibits tumor growth *in vivo*. HHD mice were immunized with control liposome, Lip-#13 or -#14 and inoculated with 2×10^4 RMA-HHD cells as indicated in Materials and methods. (A) Tumor sizes on day 24 after tumor inoculation. Tumor sizes in HHD mice vaccinated with Lip-#14 were significantly different from the control and Lip-#13-vaccinated mice (*P<0.05, Mann-Whitney U test). (B) Survival time of tumor inoculated mice. The survival time was estimated using the Kaplan-Meier method. *Statistically significant survival value between the Lip-#14 group and the control liposome group (P<0.05, Log-rank test).

used as the negative control. On day 0, we challenged HHD mice with 2×10^4 RMA-HHD lymphoma cells and monitored tumor growth and survival. The results showed that on day 24 following the challenge there was no significant difference in tumor size between Lip-#13 and the control (Fig. 5A). This result suggested that immunization with Lip-#13 did not induce sufficient protective antitumor immunity. By contrast, tumor size in Lip-#14 immunized mice was significantly lower than the control and Lip-#13 immunized mice (P<0.05, Mann-Whitney U test, Fig. 5A). Furthermore, Lip-#14 immunization prolonged the survival time of tumor-bearing mice compared with the control mice (P<0.05, Log-rank test Fig. 5B). There was no significant difference in survival time between Lip-#13 and the control (Fig. 5B). Thus, these results showed that, interference with tumor growth of TERT-expressing RMA-HHD cells was provided by immunization with Lip-#14.

Discussion

In the present study, we utilized the high homology between mTERT and hTERT (26), which enabled us to examine epitopes exhibiting high immunogenicity on the surface of liposomes and test their immunotherapeutic potency by immunizing HHD mice and challenging with RMA-HHD lymphoma cells. In addition, by including a heteroclitic variant to each epitope candidate, we identified a novel epitope #13 derived from TERT with low affinity to HLA-A2.1 molecules. The #14, the heteroclitic peptide of #13, had a high affinity to HLA-A2.1 and when coupled to the surface of liposome, induced strong and long-lasting CD8+ T cell responses against both #14 and the original epitope #13. Those responses were found to provide protection against endogenously TERT-expressing tumor cells.

As detailed characterization of many tumor cell expression molecules that act as TAAs is now available (27,28), immunotherapy has become an increasingly essential component of cancer therapies (29,30). Active cancer immunotherapy such as a cancer vaccine is based on fundamental knowledge that TAAs are presented on MHC molecules for recognition by specific T cells. However, the heterogeneous expression of most of the characterized TAAs limits the broad applicability of cancer vaccines. TAAs that can be broadly applied to various types of cancer, such as universal TAA, have been limited. Criteria for universal TAA include: i) expression by the vast majority of cancers, ii) presence of peptides that bind to MHC molecules, iii) sufficient natural antigen processing by tumor cells that leads to the expression of antigen-derived peptides in the groove of MHC, and iv) recognition by T cell repertoire in an MHC-restricted manner. Previous studies have shown TERT meets these criteria (31). From these attractive fortes, TERT has been used as target of cancer vaccine (32). However, similar to most TAA, TERT is a self-antigen that is expressed on normal tissues, including the thymus (33). This fact raises the issue of tolerance of the TERT-specific T-cell repertoire and consequently the inability to trigger a strong and efficient antitumor immune response (14). Previous reports have shown that, self antigen-specific tolerance influences mainly the T-cell repertoire against dominant self-epitopes and to a much lesser extent against the cryptic epitope (34-36). Thus, we examined the non-tolerized repertoire against low affinity epitopes of TERT as the target of the antitumor vaccination. Since low affinity epitopes may be non-immunogenic, we devised two strategies for enhancement of immunogenicity of the epitope. One was modification of the epitope sequence to enhance affinity to MHC molecules. The other was chemically coupling the epitope peptide to the surface of liposome.

It has been shown that immunization using heteroclitical peptides derived from low affinity TERT epitopes emulsified in IFA recruits non-tolerized CTL repertoire and are more efficient at inducing tumor immunity than high affinity epitopes (15). However, the #14 peptide, the heteroclitical epitope peptide identified in the present study, induced only weak CD8 T-cell immune responses against the native epitope when emulsified in IFA and injected in HHD mice. By contrast, when coupled to the surface of liposomes, it induced strong CD8 T-cell immune responses against the native cryptic epitope #13, while #13 peptide on the surface of liposomes induced poor CD8

T-cell responses. Therefore, we consider that the synergistic effect between heteroclitic modification and coupling to the surface of liposomes provided the strong immunogenicity of Lip-#14 and induced strong CD8 T cell responses against native cryptic epitope. Moreover, #1 peptide (540-548), which was reported to induce CD8 immunity against TERT when emulsified in IFA (11) had no immunogenicity when coupled to the surface of the liposomes. These findings suggest that coupling to the surface of liposomes affects immunogenicity of peptides. In a previous study, we showed that coupling to the surface of liposomes alters the immunogenicity of hepatitis C virus-derived peptides (24). The mechanisms underlying the alteration of immunogenicity of peptides by liposome coupling is unclear. We hypothesize that the difference of the immunogenicity is associated with the difference of antigen presentation mechanisms. Peptides emulsified in IFA are considered to fit into the pockets of MHC class I molecules on APCs replacing the original endogenously synthesized peptides and presented to CD8 T cells (37). On the other hand, we have shown that antigens chemically coupled to the surface of liposomes consisting of unsaturated fatty acid (oleoyl liposome) are cross-presented by APCs (4). Results from another study have shown that oleoyl liposomes may be taken up by APCs via at least two pathways: fusion with and penetration of plasma membranes and pinocytosis (38). These unique antigen presentation mechanisms may be responsible for the unique immunogenicity of the peptide antigens.

The efficient antitumor immune responses are often fraught with autoimmunity. Vitiligo-like depigmentation has been reported after immunotherapy against malignant melanoma (39). By contrast, adoptive transfer of p53-specific CTL provides p53-overexpressing tumor growth inhibition without autoimmunity (40). In the present study, we did not observe any adverse effect in liposomal antigen-injected animals despite evidence of the induction of strong TERT-specific CD8 T-cell responses. Previous findings have shown that there are differences in TERT expression in normal somatic and tumor tissues. Thus, TERT is a potential safe target for cancer immunotherapy (41). Gross et al showed that immunization with heteroclitic low affinity TERT peptide resulted in anti-TERT T-cell immunity without any adverse effect by their careful examination in various somatic tissues (15). Recent findings show the safety of immunotherapy against TERT are accumulating (15,41-43). However, future investigations should be conducted to address whether the telomerase inhibition by host immune system activated by immunotherapy can affect normal tissues.

In conclusion, vaccination with the liposome-coupled heteroclitical peptide derived from TERT elicits antitumor CD8 T-cell responses evading the influence of tolerance in HHD mice. Therefore, heteroclitical-modified antigens of cryptic epitope with low affinity for HLA molecules derived from tumor antigens coupled to the surface of liposome may play a role as an effective cancer vaccine candidate.

Acknowledgements

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