Original Articles

Chimeric peptide containing both B and T cells epitope of tumor-associated antigen L6 enhances anti-tumor effects in HLA-A2 transgenic mice

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ABSTRACT

Synthetic peptides are attractive for cancer immunotherapy because of their safety and flexibility. In this report, we identified a new B cell epitope of tumor-associated antigen L6 (TAL6) that could induce antibody-dependent cellular cytotoxicity (ADCC) in vivo. We incorporated the B cell epitope with a cytotoxic T lymphocyte (CTL) and a helper T (Th) epitope to form a chimeric long peptide. We formulated the chimeric peptide with different adjuvants to immunize HLA-A2 transgenic mice and evaluate their immunogenicity. The chimeric peptide formulated with an emulsion type nanoparticle (PELC) adjuvant and a toll-like receptor 9 agonist (CpG ODN) (PELC/CpG) induced the greatest ADCC and CTL responses. The induced anti-tumor immunity inhibited the growth of TAL6-positive cancer cells. Moreover, we observed that immunization with the chimeric peptide inhibited cancer cell migration in vitro and metastasis in vivo. These data suggest that a chimeric peptide containing both B and T cell epitopes of TAL6 formulated with PELC/CpG adjuvant is feasible for cancer immunotherapy.

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Introduction

Tumor associated antigen L6 (TAL6) is a cell surface protein of the transmembrane-4 superfamily (TM4SF) also known as TM4SF1. TM4SF proteins are over-expressed in different types of human cancers including lung, breast, colon, prostate and liver cancer [1–4]. TM4SF1-, TM4SF4- and TM4SF5-specific monoclonal antibodies can inhibit colon cancer growth, indicating that TM4SF proteins are crucial targets for cancer therapy [3,5–7]. TAL6 is over-expressed in more than 80% of human lung, breast, colon and ovarian tumors but not normal tissues [1,8,9]. Recently, TAL6 was found to play critical roles in cancer cell motility, invasion, metastasis and angiogenesis [2,8,10,11]. We previously demonstrated that an HLA-A2-restricted CTL epitope, A2-5, of TAL6 is capable of inducing CTL responses against cancer cells that express TAL6 [12]. In addition, the induced-CTL responses can be adoptively transferred to inhibit human lung cancer growth in immunocompromised mice [13].

TAL6 is an ideal antigen for cancer immunotherapy because it may be targeted by both humoral and cellular immunity. In this report, we not only identify the major B cell epitope of TAL6 but also combined it with a TAL6 CTL epitope to increase cancer killing potency. To enhance both B and T cell function, the adjuvant formulation was designed to contain both immunostimulatory molecules and an antigen delivery system. We found that TLR9 agonist CpG ODN with the emulsion type adjuvant PELC induced strong B and CTL responses against TAL6-expressing cancers in HLA transgenic mice. Furthermore, the induced anti-sera inhibited tumor cell migration and tumor metastasis to the lung.

Materials and methods

Animal and cell line

Female 6-week-old C57BL/6 mice were obtained from the National Laboratory Animal Center, Taiwan. The HLA-A2 transgenic mice were purchased from Jackson Laboratory which express HLA-A2.1 in C57BL/6 mice [14], and housed in the

Laboratory Animal Center of the National Health Research Institutes, Taiwan. All animal experiments were performed in specific pathogen-free (SPF) conditions under protocols approved by the Animal Committee of the National Health Research Institutes (NHRI).

The B16F1 cells were stably express TAL6 (B16-L6), and then were transfected with the HLA-A2 gene to generate a stable cell line B16-L6-A2. The B16-L6-A2 and MCF-7-TAL6 [12] cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin/streptomycin, 0.5 mM sodium pyruvate, 20 mM HEPES (Biological industries, Beit Haemek, Israel) at 37 °C in 5% CO_2 [12]. The EL4-L6-A2 and EL4-L6 cells were cultured in RPMI-1640 medium supplemented with 10% FBS [13].

Preparation of monoclonal antibodies

Hybridoma producing anti-TAL6 antibody (L6) was obtained from the American Type Culture Collection. 1F4 and 9C7 anti-TAL6 monoclonal antibodies were generated from BALB/c mice immunized with human TAL6 plasmid DNA as described. Monoclonal antibodies were purified by Protein A affinity chromatography using high salt conditions as described [2]. Antibody concentrations were determined by Micro BCA protein Assay Kit (PIERCE).

ELISA

For antibody epitope mapping L6, 9C7 and 1F4 Mabs were assayed by ELISA. For the assay of EP1 titers of the specific antibodies, antisera were collected from the mice immunized with EP1 peptide, and the titers of the specific antibodies were assayed by ELISA. The 96 well assay plates were coated with peptide (1 µg/ml) or cell (2×10^6). After blocking with 5% BSA-PBS, the antisera (1:1000 v/v) were diluted with 5% BSA-PBS and added to the plate for 1 h. HRP-conjugated goat anti-mouse IgG (1:4000 v/v) was used to detect EP1 antibody titer. The TMB peroxidase EIA substrate was added, which was stopped with 1N H_2SO_4. Absorbance was measured at 450 nm.

Animal study

HLA-A2 Tg mice were injected subcutaneously (s.c.) twice at a 2-week interval with peptide (50 µg/mouse) formulated in IFA [12], DOTAP liposome [15], PELC nanoparticles or TLR9 agonist CpG [16]. The CpG sequence used was 5′-TCC ATG ACG TTC CTG ACG TT-3′ with a phosphorothioate backbone. Seven days after the second immunization, the B16-L6-A2 or B16-L6 cells (2×10^4) were inoculated s.c. 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.

Antibody dependent cell-mediated cytotoxicity (ADCC)

Mice spleen cells were used as effector cells for the ADCC assay. Spleen cells were adjusted to a concentration of 8×10^6 cells/ml in LCM medium. Cells were added in tubes and then divided into aliquots (100 µl/well in 96-well plates). EL4-L6 or EL-4 target cells (2×10^7/ml) were labeled with 100 µCi of ^{51}C ($Na_2^{51}CrO_4$, PerkinElmer, MA) at 37 °C for 1 h. The ^{51}Cr-labelled EL4-L6 or EL4 cells were adjusted to a concentration of 2×10^5 cells/ml in LCM medium and then TAL6 antiserum or naïve mouse serum (1:10) was added. After 6 h, supernatants were harvested to measure the radioactivity using a gamma counter. Spontaneous release was measured in wells containing target cells alone. Triton X-100 (2%) was used to lyse the target cells to estimate maximal release. Percent cytotoxicity was determined according to the formula: percent lysis = 100 × (experimental ^{51}Cr release – spontaneous ^{51}Cr release)/ (maximal ^{51}Cr release- spontaneous ^{51}Cr release).

ELISPOT assay

HLA-A2 Tg mice were injected subcutaneously (s.c.) twice at a 2-week interval with peptide (50 µg/mouse) and TLR9 agonist CpG (10 µg/mouse) formulated in each adjuvant. At day 7 after the second vaccination, mice were sacrificed and spleen cells were collected. Spleen cells (5×10^5) were mixed with 10 µg/ml of the indicated peptides and 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 incubation, 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, the plate was washed and streptavidin-HRP (eBioscience) was added. Spots were developed using a 3-amine-9-ethyl carbazole (AEC, Sigma) solution. The reaction was stopped after 4–6 minutes by running the plate under tap water. The spots were then counted using an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

CD107a cytotoxicity assay

HLA-A2 Tg mice were injected s.c. twice with the indicated peptides (50 µg/ ml) emulsified in IFA, DOTAP or PELC in the absence or presence of CpG ODN (10 µg/ mouse). On day 7 after the second immunization, splenocytes were harvested and then resuspended to 2×10^7 cell/ml in medium that contained 10 µg/ml of the indicated peptides (50 µg/ml) or cells (2×10^6 cell/ml) and PE-conjugated anti-mouse CD107a antibody (1:100) in 96-well round-bottom plates. After 2 hours at 37 °C, brefeldin A (10 µg/ml) and monensin (0.66 µg/ml) were added for another 2–6 hours. The plates were washed with PBS containing 0.1% FBS, 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 detected using flow cytometer (FACS Calibur, BD Bioscience) and data were analyzed by FlowJo software.

Wound healing assay

Wound healing was investigated using a Culture-Insert (500 µm) (Ibidi). A 100 µl suspension of B16-L6 cells in DMEM-10% FBS (5×10^6 cells/ml) was seeded into each well of the insert. After cell attachment for 24 h, the culture inserts were removed and the cells were incubated with antiserum (1:100 v/v) in DMEM-10% FBS. The cell migration into the defined cell free gap was observed for 48 h as indicated under an inverted microscope. For assay analysis, cells were tracked using the manual tracking software component of the ImageJ programmer.

Statistical analysis

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

Results

Identification of B cell epitopes of TAL6

Three purified anti-TAL6 monoclonal antibodies (1F4, 9C7, L6) [2] were used to detect the surface TAL6 expressed on EL-4 (EL-4-L6) cells. Serial diluted monoclonal antibodies bound EL-4-L6 cells but not negative control EL-4 cells (Fig. 1B), confirming that these three monoclonal antibodies recognize an extracellular domain of TAL6. To further map the antibody-binding epitopes, five peptides (EL1 and EP1-4) that cover the EL1 and EL2 extracellular loops were used to determine the linear B cells epitopes using ELISA [6]. Monoclonal antibody 1F4 and 9C7 cannot recognize these peptides. By contrast, EP1 peptide could be detected by L6 mAb (Fig. 1C). To test if the EP1 peptide can raise antibodies that bind native TAL6, the EP1 peptide or MCF-7-L6 cells (as positive control) were formulated with IFA/Th and immunized into C57BL/6 mice. Sera from immunized mice were collected and the anti-TAL6 antibody titers were analyzed using cell-based ELISA. The sera (1:500) of mice immunized with EP1 or MCF-7-L6 cells had significantly higher amount of antibody than mice immunized with vehicle (PBS) (Fig. 2A). We further investigated whether EP1 immunization could induce antibody-dependent cellular cytotoxicity (ADCC). Sera (1:100) from mice immunized with either EP1 or MCF-7-L6 cells could kill TAL6-expressed EL4 (EL4-L6) cells but not EL4 parental cells (61.82% ± 6.12% vs. 0.36% ± 0.96% and 65.54% ± 1.77% vs. 11.41% ± 3.094%, Fig. 2B). To investigate if the EP1 peptide could induce anti-tumor activity, mice were immunized with EP1 peptide and then challenged with B16-L6 cells (2×10^4/mouse) at 7 days after the final immunization. Immunization of mice with either EP1 or MCF-7-L6 cells significantly inhibited tumor growth (Fig. 2C). We conclude that the EP1 peptide is a linear B cell epitope of TAL6 that can produce antitumor activity against cancer cells that express TAL6.

A chimeric peptide containing B and T cells epitopes induces greater anti-tumor activity than B or T cell epitopes alone

To investigate whether incorporation of a cytotoxic T cells (Tc) epitope and a Helper T epitope (Th) can enhance the anti-tumor effects of B-cell epitope-based approaches, the synthetic peptides Th-A2-5, Th-EP1 or Th-A2-5-EP1 (Table 1) were formulated with incomplete Freund's adjuvant (IFA) and immunized in HLA-A2 transgenic (Tg) mice. Seven days after the final immunization, mice
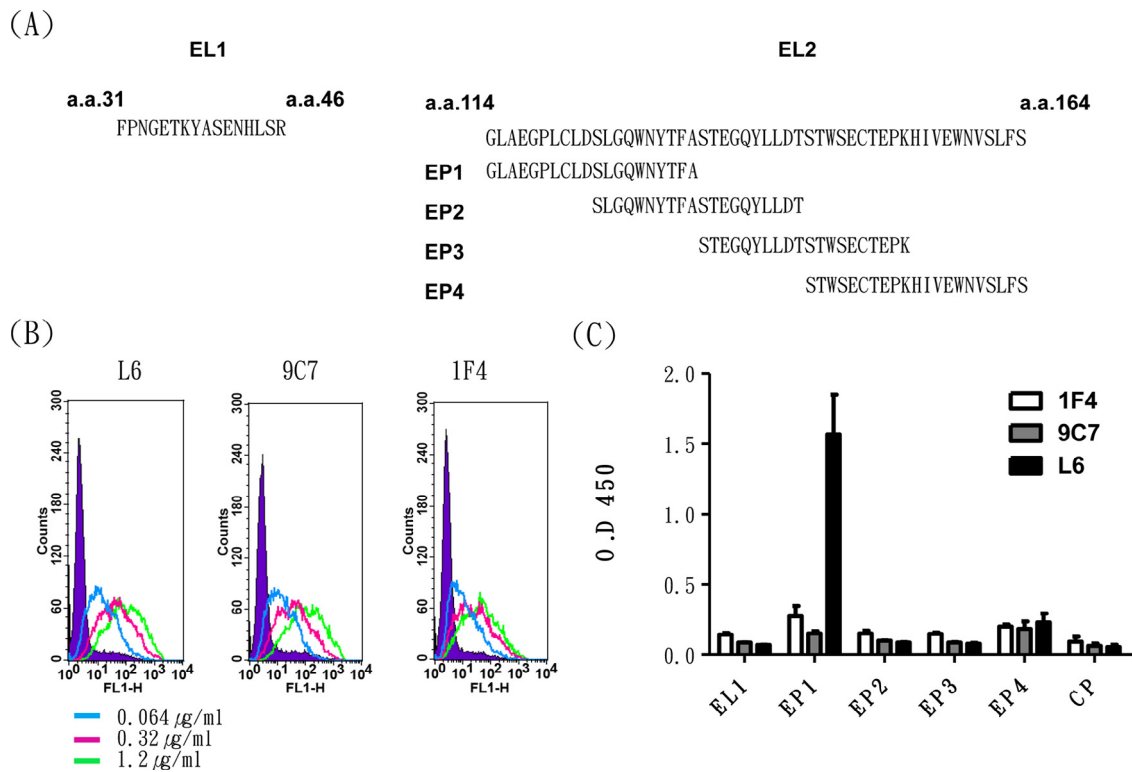
(A)

EL1   **EL2**

a.a.31   a.a.46   a.a.114   a.a.164

    FPNGETKYASENHLSR   GLAEGPLCLDSLGQWNYTFASTEGQYLLDTSTWSECTEPKHIVEWNVSLFS

EP1   GLAEGPLCLDSLGQWNYTFA

EP2   SLGQWNYTFASTEGQYLLDT

EP3   STEGQYLLDTSTWSECTEPK

EP4   STWSECTEPKHIVEWNVSLFS

(B)

L6   9C7   1F4

— 0.064 μg/ml
— 0.32 μg/ml
— 1.2 μg/ml

(C)

Fig. 1. Identification of a linear B-cell epitope by peptide mapping. (A) The amino acid sequence (EL1: a.a.31-a.a.46; EL2: a.a.114-a.a.164) of the major extracellular domains of TAL6. The overlapping peptides EP1-EP4 cover the second extracellular loop of TAL6. (B) Three anti-TAL6 monoclonal antibodies were used to detect TAL6 expression (EL-4-L6) using flow cytometry. (C) A 96-well plate was coated with the indicated peptides (10 μg/ml) in each well. Three anti-TAL6 antibodies (1:500) were used to detect these peptides. CP: Control peptide.
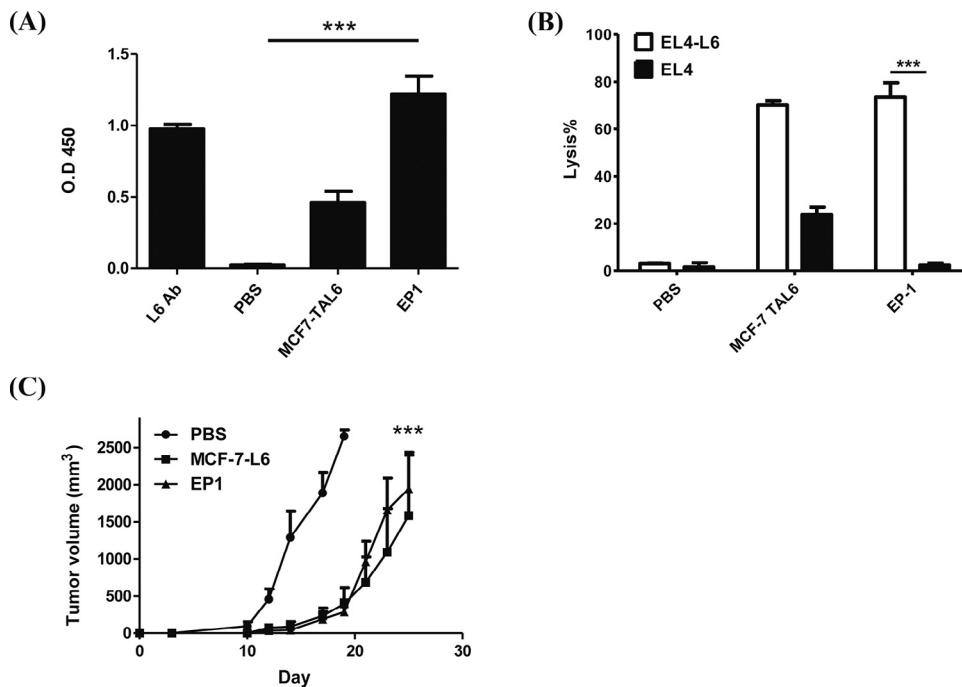
(A)   ***

(B)   ***

(C)

Fig. 2. Immunization of the EP1 peptide induces anti-tumor immunity in WT mice. The EP1 or positive control MCF-7-L6 cells formulated with incomplete Freund's adjuvant (IFA) were immunized in mice twice at a two week interval. (A) Anti-TAL6 antibody titers were determined by using cell-based ELISA. Antigen-specific antibody was calculated as: EL4-L6 (O.D.) – EL4 (O.D.). (B) TAL6-specific ADCC was determined in a Cr^{51} release assay. The lysis percentage was calculated as: immunized serum (lysis %) – naïve serum (lysis%). ***P < 0.001. (C) At 7 days after the final immunization, B16-L6 (2×10^4) cells were inoculated subcutaneously. The tumor growth was monitored 2–3 times per week. (D) The survival rate of mice is showed.

Table 1
Amino acid sequence of the synthetic peptides.

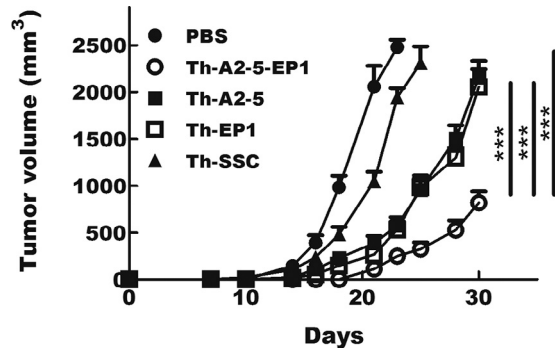| Name	Sequence
A2-5	LLMLLPAFV
EP1	GLAEGPLCLDSLGQWNYTFA
Th	AKFVAAWTLKAAA
Th-A2-5	AKFVAAWTLKAAAAAALLMLLPAFV
Th-EP1	AKFVAAWTLKAAAAAAGLAEGPLCLDSLGQWNYTFA
Th-A2-5-EP1	AKFVAAWTLKAAAAAALLMLLPAFVAAAGLAEGPLCLDSLGQWNYTFA
Th-SCC	AKFVAAWTLKAAASSCSSCPLSKI

Fig. 3. Combination of T and B cell epitopes induces anti-tumor activity in HLA-A2 mice. Peptides formulated in incomplete Freund's adjuvant (IFA) were used to immunize HLA-A2 mice twice at a two week interval. At 7 days after the final immunization, B16-L6-A2 cells (2×10^4) were injected subcutaneously. The tumor size was monitored 2–3 times per week. Tumor volume = length × width × width/2.

were challenged with B16-L6-A2 cells. Immunization with the chimeric Th-A2-5-EP1 peptide inhibited tumor growth whereas Th-A2-5 or Th-EP1 peptides did not provide significant antitumor activity (Fig. 3). The induction of both humoral and cellular immunity using chimeric peptide may synergize the anti-tumor activity.

Chimeric peptide formulated with PELC/CpG induces strong humoral and cellular anti-tumor immunity

We further tested different adjuvant formulations to optimize the anti-tumor activity of Th-A2-5-EP1. The adjuvant IFA, DOTAP liposomes with or without the TLR9 agonist ODN CpG (CpG), and PELC emulsion type adjuvant with or without CpG were used to formulate Th-A2-5-EP1. The Th-A2-5-EP1 peptide formulated with PELC containing CpG induced the highest IgG antibody amount against EP1 (O.D. 1.41 ± 0.04) (Fig. 4A). The PELC adjuvant induced higher antibody than the IFA adjuvant (O.D. 1.01 ± 0.24 vs. 0.622 ± 0.183). Th-A2-5-EP1 peptide formulated with DOTAP liposomes, DOTAP/CpG or CpG alone produced similar level of antibody (O.D. 0.454 ± 0.025, 0.46 ± 0.02 and 0.54 ± 0.02, respectively).

Sera from Th-A2-5-EP1 in different adjuvants immunized mice were used to evaluate ADCC activity. The Th-A2-5-EP1 peptide formulated with PELC/CpG induced the highest level of ADCC (Fig. 4B). The Th-A2-5-EP1 peptide formulated in the PELC adjuvant induced the second highest ADCC activity. These results correlated with the anti-EP1 antibody titers.

Our previous report showed that A2-5 formulated with ISA/CpG induced anti-tumor activity against TAL6-positive cancer cells
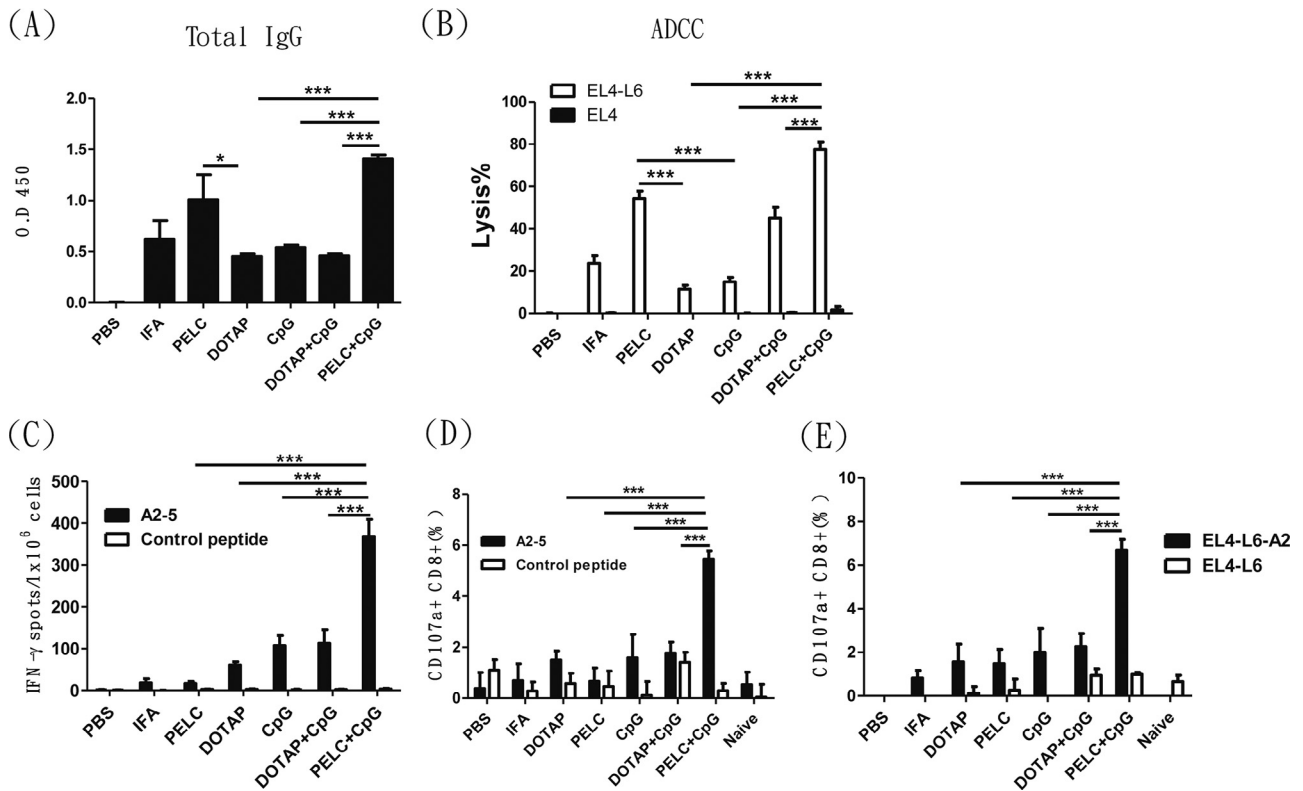

Fig. 4. Immunization of Th-A2-5-EP1 induces humoral and cellular immunity against TAL6-positive cancer. The Th-A2-5-EP1 peptide was formulated with different adjuvants and immunized in HLA-A2 transgenic mice twice at a 2 week interval. (A) Anti-EP1 antibody titers were determined by ELISA. *P < 0.05. ***P < 0.001. (B) TAL6-specific ADCC was determined by Cr^{51} release assay. The lysis percentage was calculated as: immunized serum (lysis %) – naïve serum (lysis%). ***P < 0.001. (C) Splenocytes were harvested from peptide immunized mice and stimulated with 10 μg/ml of EP1 peptides or control peptide for 48 h. IFN-γ-secreting cells were determined using IFN-γ ELISPOT assay. Results are expressed as mean + SD. ***P < 0.001. (D) Splenocytes were harvested from peptide immunized mice and stimulated with 10 μg/ml of EP1 peptides or control peptide for 6 h in the presence of PE-conjugated anti-CD107a. After stimulation, FITC-conjugated anti-CD8 antibody was used to detect CD8$^+$ T cell. The percentage of CD107a$^+$ CD8$^+$ cells in individual immunized groups was determined by flow cytometry. (E) Irradiated EL4-L6-A2 or EL4-L6 cells (2×10^4) were used to stimulate splenocytes for 2 h. The percentage of CD107a$^+$ CD8$^+$ cells was determined by flow cytometry. ***P < 0.001.
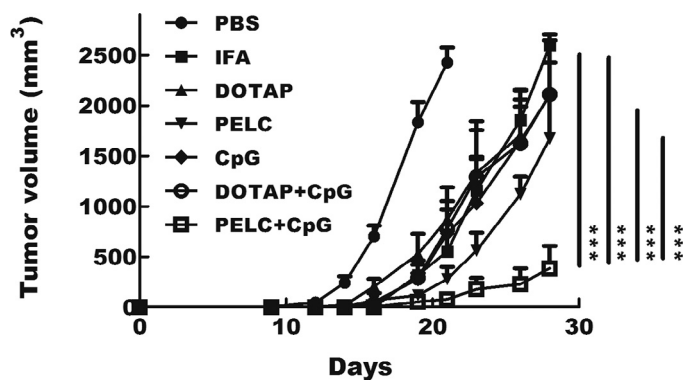
Fig. 5. Immunization of Th-A2-5-EP1 induces anti-tumor activity in HLA-A2 transgenic mouse. HLA-A2 transgenic mice were immunized with Th-A2-5-EP1 peptide formulated in different adjuvants twice. At 7 days after the final immunization, 2×10^4 of B16-L6-A2 cells were injected subcutaneously. Each group contains 6 mice. (A) The tumor growth was monitored 2–3 times per week. Results are expressed as mean +SD.

[12,13]. To investigate whether the Th-A2-5-EP1 chimeric peptide can induce A2-5-specific T cell responses, IFN-γ ELISPOT and CTL activity assays were performed. Fig. 4C shows that Th-A2-5-EP1 formulated with PELC/CpG induced higher numbers of spots (368.5 ± 40.42) than the peptide in other adjuvant formulations. A significant increase in the number of spots as compared to control mice were detected when Th-A2-5-EP1 peptide was formulated with DOTAP (60.5 ± 7.85), CpG (107.2 ± 23.89) or DOTAP/CpG (112.5 ± 33.18) but not PELC (16.7 ± 4.74) or IFA (18 ± 9.75). We conclude that Th-A2-5-EP formulated with PELC/CpG is capable of inducing strong A2-5-specific T cell responses. In contrast to the antibody titer, Th-A2-5-EP1 peptide formulated with PELC did not induce significant numbers of IFN-γ-secreting cells (Fig. 4A and C).

To further determine if the PELC/CpG formulation can induce cytotoxic activity, the number of CD107a+CD8+ T cells was analyzed. Splenocytes from various groups were stimulated with A2-5 or irrelevant control peptide (10 µg/ml) for 6 hours. Th-A2-5-EP1 peptide formulated with PELC/CpG induced higher numbers of CD107a+CD8+ T cells ($5.44 \pm 0.35\%$) than did the other adjuvant formulations (Fig. 4D). To confirm that the induced CTL responses are HLA-A2-dependent, splenocytes from the various groups were stimulated with EL4-L6-A2 or EL4-L6 cells for 6 hours. Accordingly, Th-A2-5-EP1 formulated with PELC/CpG induced higher numbers of CD107a+CD8+ T cells ($6.69 \pm 0.49\%$) than did the other adjuvant formulations (Fig. 4E). These results demonstrate that Th-A2-5-EP1 formulated with PELC/CpG could induce strong A2-5-specific CTL responses in A2 Tg mice.

Because the B16-L6-A2 cell line is an aggressive cancer model, it is not easy to control tumor growth using therapeutic regimen, so we used prophylactic vaccination to determine their efficacy. To evaluate the anti-tumor effects of Th-A2-5-EP1 in different adjuvant formulations, HLA-A2 Tg mice were challenged with B16-L6-A2 cells (2×10^4) at 7 days after the second immunization. Th-A2-5-EP peptide formulated with PELC/CpG significantly inhibited tumor growth as compared to the other formulations. Th-A2-5-EP peptide formulated with IFA, DOTAP, PELC, CpG or DOTAP/CpG displayed moderate anti-tumor activity (Fig. 5). We conclude that a long peptide containing Th, Tc and B cells epitope formulated with PELC/CpG could induce strong humoral and cellular immunity against cancer.

Chimeric peptide Th-A2-5-EP1 formulated with PELC/CpG inhibits tumor migration and metastasis

Because TAL6 has been shown to be involved in cancer cell migration and metastasis [2,10], we examined whether the sera from Th-A2-5-EP1 immunized mice could inhibit cancer cell migration. A chambers wound assay was performed to evaluate the inhibition of cell migration. B16-L6 cell were cultured in 24-well plate with a CultureInsert (500 µm) for 24 hours, and then the Culture-Insert was removed and 100 µl of medium, sera (1:100, v/v) or positive control (L6 mAb) were added. Fig. 6A shows images acquired at 0, 24 and 48 hours. Obvious inhibition of cancer cell migration was observed after treatment with either L6 or serum from
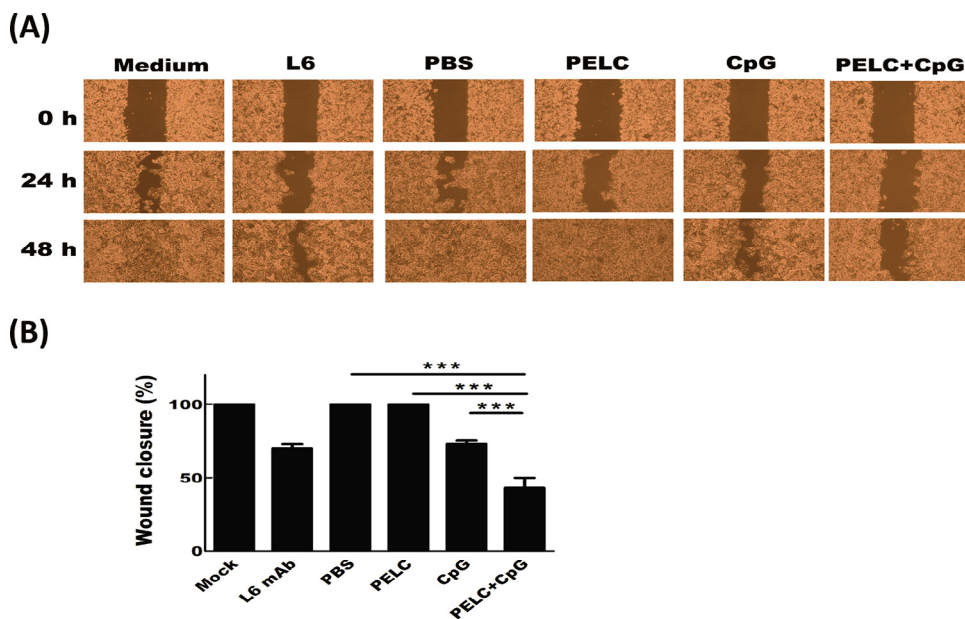

Fig. 6. Sera from immunized mice inhibit cancer cell migration. The B16-L6 cells were cultured in a 24-well plate which contained one Culture-Insert (500 µm) in each well. After 24 h, the Culture-Inserts were removed and 500 µl of medium with or without antisera (1:100, v/v) from immunized mice was added. Images were acquired at 0, 24 and 48 h. L6 mAb (10 µg/ml) was used as a positive control. The cell migration into the defined cell free gap was observed at the indicated times under a microscope. (A) Representative picture; (B) data are shown as mean ± SD (n = 5). Migration (%) = gap area at 48 h/gap at 0 h × 100%. ***P < 0.001.
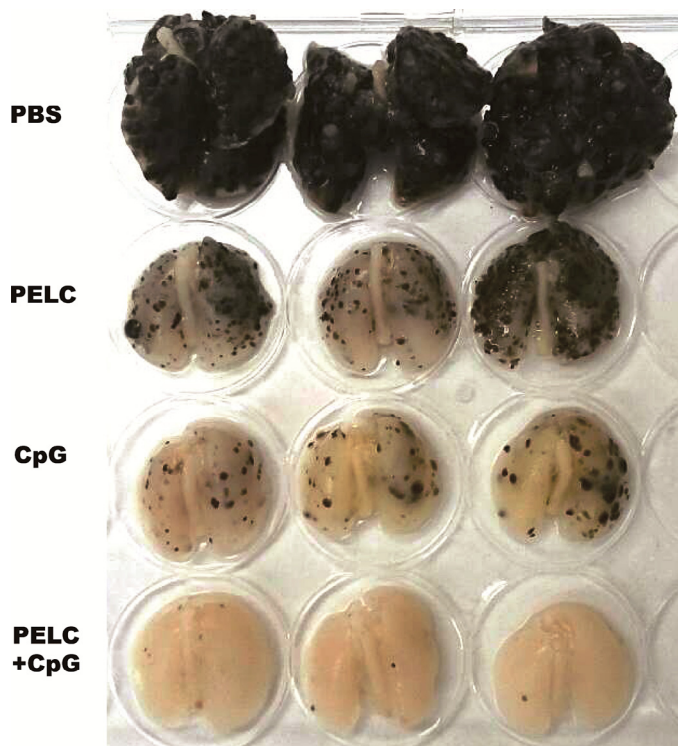
Fig. 7. Chimeric peptide formulated with PELC and CpG inhibits B16-L6-A2 lung metastasis. HLA-A2 mice were immunized with Th-A2-5-EP1 (50 μg/mouse) in PELC twice at a two week interval. At 7 days after the final immunization, B16-L6-A2 cells (5×10^5) were injected intravenously. At 20 days after cell inoculation, mice were sacrificed and lung tissues were collected to observe the formation of tumor nodules.

mice immunized with Th-A2-5-EP1 and PELC/CpG. Quantification of the cell-free gap in three independent experiments revealed that sera from mice immunized with CpG alone ($72.97\% \pm 5.97$) or PELC/CpG ($43.04\% \pm 15.47$) significantly inhibited the B16-L6 cell migration. Migration inhibition was not due to differences in cell proliferation (Fig. S1).

Inhibition of cancer cell migration suggested that cancer cell metastasis might also be inhibited by immunization with chimeric peptide. The anti-metastasis activity of peptide immunization was examined in a B16-L6-A2 metastasis model. The Th-A2-5-EP1 peptide formulated with PELC, CpG or PELC/CpG was used to immunize mice twice at a two week interval. Seven days after the second immunization, B16-L6-A2 cells (5×10^5) were injected intravenously. At 22 days after cancer cells inoculation, the mice were sacrificed and lung tissues were collected. Th-A2-5-EP1 formulated with PELC, CpG or PELC/CpG dramatically reduced tumor nodule formation in lungs (Fig. 7). Interestingly, almost no tumor nodules were observed in mice immunized with Th-A2-5-EP1 peptide with PELC/CpG. We conclude that Th-A2-5-EP1 formulated with PELC/CpG could inhibit B16-L6-A2 cancer cell lung metastasis.

Discussion

Here we identified a B-cell epitope of TAL6 that induced anti-tumor activity in the presence of adjuvant. Combination of Th (pan-DR) and Tc (HLA-A2-restricted) epitopes with the B-cell epitope in a long peptide enhanced the anti-tumor activity in HLA-A2 Tg mice. HLA-A2 Tg mice are a good model for the evaluation of anti-tumor effects on HLA-A2-positive human cancer and more closely related to the clinical setting. Importantly, the chimeric peptide elicited strong humoral and cellular immunity against tumor growth and metastasis when formulated with PELC/CpG. Our findings are

feasible to translate into clinical trials for immunotherapy of TAL6-positive cancers.

TAL6 has long been a target for monoclonal antibody therapy. Most TAL6 antibodies recognize the extracellular loop 2 (EL2) [6,17]. However, there are no reports that identified the linear B cell epitope of EL2. Although B-cell epitope prediction software can be used to predict potential antigenic epitopes [http://tools.immuneepitope.org/bcell/; http://www.dnastar.com/t-sub-solutions-structural-biology-epitope-prediction.aspx; http://sysbio.unl.edu/SVMTriP/], the use of monoclonal antibodies to identify B cell epitopes is more certain. We tested three anti-TAL6 monoclonal antibodies that recognize the extracellular domain of TAL6, but found that only one of the monoclonal antibodies can recognize a linear epitope EP1 (Fig. 1). EP1 immunization induced functional antibodies to kill TAL6-positive cancer cells in vitro and in vivo (Fig. 2). ADCC is an important mechanism for monoclonal antibodies to kill cancer cells. The mAb L6 has been reported to induce ADCC against human lung cancer cell lines [9]. EP1 immunization-induced anti-sera also killed TAL6-positive cells through ADCC (Fig. 2B). Although a chimeric L6 antibody has been conjugated with radio isotopes [18,19] or enzymes for pro-drug activation [20,21] to treat cancer in animals, these products did not move to market. These monoclonal antibodies alone or conjugated with cytolytic reagents may not completely kill all cancer cells or their variants. TAL6-specific T cell immunity may therefore enhance anti-tumor activity. In this report, we combined both Th and Tc epitopes with EP1 to enhance therapeutic activity against TAL6-positive cancers.

Synthetic peptides as immunogens are safe but low immunogenicity. To improve their efficacy, incorporation of potent adjuvants is critical. Incomplete Freund's Adjuvant and DOTAP liposome have been tested in clinical studies [22]. Synthetic peptides formulated with DOTAP liposome have been shown to induce anti-tumor immunity [15,23]. CpG can enhance the anti-tumor activity of liposomal peptides [24]. We found that formulation of the chimeric peptide with PELC/CpG was more potent than a DOTAP liposome/CpG formulation in the induction of both B and T cell immune responses (Fig. 4). Our previous studies showed that ovalbumin formulated with PELC induced higher levels of antibodies but not T cell responses as compared to formulation with alum [25]. Similar results were observed for a recombinant dengue viral envelop protein domain II formulated with PELC [26]. Accordingly, peptide formulated with PELC induced higher antibody titers than peptide formulated with IFA. However, PELC did not induce greater T cells responses (Fig. 4). The TLR9 agonist CpG directly induces T-helper 1 biased immune responses in mice [27]. Some pre-clinical studies demonstrated that CpG improves the anti-tumor activity of immunotherapeutic reagents [28–30]. Our studies showed that a CTL epitope of HPV16 E7 protein formulated with PELC and CpG additively enhanced CTL responses and anti-tumor effects [16]. Peptide mixed with only CpG did not display significant enhancement of anti-tumor activity. The enhanced anti-tumor activity of PELC/CpG may be due to the slow-release of antigen and CpG. In the present study, Th-A2-5-EP1 formulated with PELC/CpG induced high levels of A2-5-specific CTL responses but moderate CTL levels when formulated with PELC or CpG alone (Fig. 4D). The combination of both emulsion type adjuvant PELC and immunostimulatory adjuvant CpG is able to synergize anti-tumor immunity. We further confirmed the induced CTL responses are HLA-A2-restricted because the CTL responses were observed against EL4-L6-A2 but not EL4-L6 (Fig. 4E). Our previous data showed that A2-5-induce CTL in HLA-A2 Tg mice could inhibit human cancer xenografts in immune compromised mice [13]. Taken together, our results indicate that Th-A2-5-EP1 immunization-induced CTL can kill TAL6-positive human cancer cells.

In addition to the direct killing function of effector cells, inhibition of tumor migration or angiogenesis may play important roles

in cancer therapy. CD13 can associate with TAL6 on cancer cells and enhance cancer cell migration. Monoclonal anti-TAL6 antibodies have been shown to inhibit lung cancer cell invasion and migration [2]. The Th-A2-5-EP1 peptide in CpG or PELC/CpG formulations inhibited cancer cell migration in a wound-healing assay. Interestingly, the high antibody titers induced by the PELC formulation could not inhibit tumor migration whereas the low antibody titers induced by the CpG formulation did. We further analyzed the IgG antibody isotypes induced by the various formulations. We found that the PELC formulation induced high levels of IgG_1 antibodies but low levels of IgG_{2a} and IgG_{2b} antibodies (Fig. S2). By contrast, the CpG formulation induced high levels of IgG_{2b} antibodies but low levels of IgG_1 antibodies. We speculate that the IgG_{2b} antibody may have potent migration-inhibition activity. However, we cannot exclude that the migration-inhibition activity is due to the quality of the antibodies produced by the different formulations. The in vitro observations were confirmed in an in vivo lung metastasis model. Immunization of mice with the CpG formulation resulted in fewer tumor nodules in lungs as compared with the PELC formulation (Fig. 7). In addition to the migration-inhibition effects of the antibodies, complement-mediated cytotoxicity or ADCC may also contribute to the reduction of tumor nodules in the lungs. Based on our data, immunization with PELC or CpG formulations induced similar levels of ADCC in vitro and inhibition of subcutaneous tumor growth in vivo. These results demonstrate that immunization with the CpG formulation can provide other anti-tumor functions (i.e. cancer cells migration-inhibition) to reduce tumor nodules in the lungs.

Most peptide-based vaccines are focused on the induction of CTL responses [31–34]. The generation of CTL responses is correlated with the prevention or eradication of tumor cells in animal models, but limited efficacy is often observed in humans. In particular CTL responses may not well control cancer metastasis [32]. These results indicate that CTL responses alone are not sufficient to control tumor progression. Here, we show that induction of both antibody and CTL responses against TAL6 not only eliminated cancer cells but also inhibited tumor migration or invasion. The two-armed immunization approach is an ideal design to target tumor-associated antigens which are expressed on the surface of cancer cells. To achieve the successful peptide-based cancer immunotherapy, the selection of potent adjuvants to induce functional anti-tumor activity is another key. Our data strongly support the idea that peptides containing both B and T cells epitopes in PELC/CpG formulation could be applied to other targets (i.e. Her2/Neu).

Acknowledgments

Conflict of interest

The authors have no conflict of interest to declare.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.04.031.

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