A novel emulsion-type adjuvant containing CpG oligodeoxynucleotides enhances CD8+ T-cell-mediated anti-tumor immunity

Ying-Chyi Song a, Han-Yin Cheng a, Chih-Hsiang Leng a,b, Sheng-Kuo Chiang a, Chih-Wei Lin a, Pele Chong a,b, Ming-Hsi Huang a,**, Shih-Jen Liu a,b,**

a National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Miaoli, Taiwan
b Graduate Institute of Immunology, China Medical University, Taichung, Taiwan

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A B S T R A C T

PELC is a novel emulsion-type adjuvant that contains the bioresorbable polymer poly (ethylene glycol)-block-poly (lactide-co-e-caprolactone) (PEG-b-PLACL), Span®85 and squalene. To investigate whether PELC is able to enhance CTL responses of antigens for treating tumor, peptides or protein antigens derived from HPV16 E7 were formulated with PELC nanoparticles and CpG oligodeoxynucleotide. We identified that PELC formulation could delay the release of antigens in vitro and in vivo. We assessed the immunogenicity of an H-2D b-restricted CTL epitope RAHYNYIVTF (RAH) formulated with PELC or PELC/CpG and investigated the ability of these formulations to promote tumor regression. Following a single-dose subcutaneous injection in mice, we found that the RAH peptide formulated with PELC/CpG (RAH/PELC/CpG) resulted in increased numbers of IFN-γ-secreting cells and RAH-specific CD8+ T cells and an enhanced cytotoxic T cell response compared with RAH formulated with PELC or CpG alone. The tumor-bearing mice received a single-dose injection of RAH/PELC/CpG, which induced complete tumor regression. These results demonstrated that peptide antigen formulated with PELC/CpG nanoparticles is feasible for cancer immunotherapy.

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1. Introduction

Human papillomavirus (HPV) infection is the most common cause of cervical cancer. Over 100 HPV types have been identified; some of the types that have been detected in cervical cancer are called “high-risk” HPV [1]. The four HPV types most frequently found among women worldwide with cervical cancer are types 16, 18, 33 and 58. Currently, there are two protein-based HPV vaccines (Cervarix® for types 16 and 18 and Gardasil® for types 6, 11, 16 and 18) used to prevent HPV infection and cervical intraepithelial neoplasia [2–7]. Both vaccines contain the HPV capsid protein (L1), which can induce neutralizing antibodies that block virus infection but cannot destroy virally infected or cancer cells. However, the high cost of the HPV vaccines limits mass vaccination in developing countries. Therefore, the development of efficient therapeutic approaches for cervical cancer is still needed. The HPV E7 oncoprotein is highly conserved among different genotypes [8–9]; moreover, it is a tumor-specific and a tumor-rejection antigen. Therefore, some studies have designed the E7 protein as the target for the development of therapeutic vaccines for treating HPV16-associated cancer and its precursor lesions [10–12]. In addition, the evolution of vaccine strategies has seen a move from whole organisms to recombinant proteins, with further development towards the most minimal epitope possible. Nonetheless, a major challenge remains to turn poor immunogenic epitopes into potent and effective vaccines.

In general, vaccines contain adjuvants to enhance the immunity against the immunogens and to thereby make the vaccine more effective. Aluminum-based mineral salts are currently incorporated as adjuvants in prophylactic HPV vaccines (Cervarix® and Gardasil®). These adjuvants effectively induce protective antibody titers against virus entry but do not induce Th1 responses or CTLs for killing HPV-infected or cervical cancer cells [13]. To this end, many new adjuvant formulations are being examined in various preclinical and clinical trials for treating cervical cancer [14]. On the basis of their mechanisms of action, adjuvants can be broadly divided into delivery systems and immunopotentiators [15,16]. The delivery systems (for example, liposomes and MF59) may target antigens to the key cells and/or sites necessary to colocalize antigens and immunopotentiators [17,18]. In our previous study, we introduced a new delivery system adjuvant (PELC) for the development of vaccines that was a water-in-oil-in-water nanoemulsion composed of a bioresorbable polymer, Span®85, and squalene [19]. In
this emulsion, squalene was selected as the core oil, bioreersorbable poly (ethylene glycol)-block-poly (lactide-co-o-caprolactone) (PEG-b-PLACL) served as a hydrophilic emulsifier, and Span®85 acted as a hydrophobic emulsifier to stabilize the water/squalene interface. We also showed that a single-dose injection of the PELC nanoparticle-formulated inactivated HSN1 virus vaccine in mice induced potent antigen-specific antibody titers [20]. Moreover, emulsified PELC nanoparticles containing inactivated an HSN1 virus and CpG (an immunostimulatory oligodeoxynucleotide containing unmethylated cytosine–guanosine motifs) induced a crossprotecting antibody response against a heterologous virus strain [21]. However, whether the peptide/protein formulated with the PELC nanoparticle can induce cytotoxic T lymphocyte responses still needs to be investigated.

Immunopotentiators activate innate immunity directly (for example, cytokines) or through pattern-recognition receptors (PRRs, such as those for bacterial components). The Toll-like receptors (TLRs) are a family of PRRs that are an important link between innate and adaptive immunity. Some studies have shown that TLR ligands have adjuvant activity and enhance antigen-specific antibody and cell-mediated immune responses, especially when they are combined with delivery systems that promote their uptake and delivery into antigen-presenting cells [22–24]. For clinical studies, TLR9 is generally stimulated with synthetic oligodeoxynucleotides containing one or more unmethylated CpG dinucleotides. In humans, CpG has been used as an adjuvant for infectious disease vaccination [25,26] and in the development of cancer therapy [27]. In a mouse model, CpG has also been shown to induce Th1 helper 1 (Th1) immune responses, which are characterized by the production of IFN-γ and the generation of IgG2a [28,29]. Moreover, a previous study had demonstrated that different liposomas with CpG ODN significantly increased Th1-biased cytokines and augmented cell mediated immune response [30].

For this article, we selected an H-2Db-restricted CTL epitope (amino acids 49–57, RAHYNIVTF) (RAH) derived from the HPV 16 E7 protein or the recombinant mutant E7 (rE7m) [31] as models to study the cellular immune responses to the peptide/protein formulated with PELC or with a combination of PELC and CpG and to evaluate whether the candidate vaccine formulations could be used as a tool for cancer immunotherapy. We examined the cellular immune responses and the tumor regression in mice that received a single injection of RAH peptides alone or of RAH peptides formulated with PELC, CpG, or a PELC/CpG combination. This study demonstrated that peptide antigens formulated with both a delivery system (PELC) and an immunopotentiator (CpG) could induce strong CTL responses against cervical cancer.

2. Materials and methods

2.1. Materials

The H-2Db-restricted CTL epitope (amino acids 49–57, RAHYNIVTF) (RAH) derived from the HPV16 E7 protein was synthesized in-house by the solid phase method using an automated peptide synthesizer, model PS-3 from Protein Technologies, Inc. (Tucson, AZ, U.S.A.), employing the fluorenylmethoxy carbonyl (Fmoc) group for α-amino group protection. The final de-blocking step was carried out with Reagent K (a mixture of TFA/thioanisole/phenol/water/EDT). The crude peptide was recovered by a precipitation method with ether as the non-solvent and further purified by reversed-phase HPLC using a gradient elution. Mass spectrometry data were obtained from an Agilent 1100 Series LC/MSD highperformance ion trap mass spectrometer to ensure that purity higher than 90% was obtained. The peptide was dissolved in DMSO (Sigma, MO, USA) at a stock concentration of 10 mg/mL. CpG ODN was synthesized by Invitrogen Taiwan Ltd. and given as a 10 μg dose dissolved in PBS or in the antigenic media. The CpG ODN sequence used was 5’-TCC ATG ACG TTC CTG TT-3’ with a phosphorothioate backbone. The TC-1 cell line, a mouse epithelial cell line transformed with the oncogenes Ras and HPV16 E6 and E7, was a kind gift from Dr. T-C. Wu (Johns Hopkins University, USA). TC-1 cells were cultured in DMEM (GIBCO-BRL, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO-BRL, NY, USA). A complete RPMI-10 medium contained RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 25 mM HEPES (Biological industries, Beit Haemek, Israel), penicillin (100 U/ml) and streptomycin (100 μg/ml).

2.2. The preparation of PELC

PELC is a squalene nanoemulsion stabilized by Span®85 (sorbitan trioleate, Sigma-Aldrich, Steinheim, Germany) and PEG-b-PLACL, as previously described [19,20,32]. Briefly, 120 mg of PEG-b-PLACL, 0.8 ml of phosphate buffered saline (PBS), and 1.1 ml of an oily solution consisting of squalene (Sigma-Aldrich, Steinheim, Germany) and Span®85 (85/15, v/v) were emulsified using a Polytron®PT 3100 homogenizer (Kinematica AG, Switzerland) at 6000 rpm for 5 min. The emulsified PELC formulation was stored at 4 °C until use. A PELC-formulated vaccine was prepared for experiments by re-dispersing 200 μl of a stock emulsion into 1800 μl of a bulk vaccine and mixing with a test tube rotator (Labinco LD-79, Netherlands) at 5 rpm at least 1 h before injection. The microscopic aspects of the emulsions were monitoring with an optical microscope (Olympus DP70 Digital Microscope Camera, Melville, NY, USA). Particle size distribution was determined by the laser light scattering technique. Peptide release experiments were performed with the inverted dialysis tube method [33]. Briefly, RAH-containing formulations (50 μg per 50 μl) were first placed in a dialysis chamber (cut-off 0.2 μm), and then the device was immersed in a 1.5 ml eppendorf containing 1 ml ddH2O and placed at 37 °C. At different time intervals, the RAH release was regularly determined by the micro bicinchoninic acid (BCA) method (MicroBCA™ protein assay kit; Thermo, IL, USA).

2.3. In vivo fluorescence imaging

Alexa Fluor® 647-conjugated RAH peptide (RAH-Alexa 647, absorption = 650 nm; fluorescence emission = 668 nm) was purchased from GeneDireX (Nevada, USA). C57BL/6 mice were subcutaneously injected with 50 μg of RAH-Alexa 647 combined with CpG (10 μg), PELC (10%) or both. Before using Spectrum Imaging System, mice were anesthetized with isoflurane at a maintenance concentration of 2.5%, and oxygen pressurized at 4 kg/cm² in conjunction with XGI-8 Anesthesia System. After treatment, in-life fluorescence analysis was performed at 6, 30, 48, 72, 144, 168, 240, 336 and 384 h by using a Xenogen IVIS® Spectrum 200 Imaging System (Caliper Life Sciences, USA). The fluorescent measurement and region of interest (ROI) were quantified and analyzed by using IVIS Living Image 4.0 software package.

2.4. Animals

Female C57BL/6 mice, 6–12 weeks of age, were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). All the animals were housed at the Animal Center of National Health Research Institutes (NHRI) and maintained in accordance with the institutional animal care protocol. All of the animal studies were approved by the animal committee of the NHRI (NHRI-IACUC-095-051-A).

2.5. The detection of peptide-specific CD8+ T cells

The C57BL/6 mice were immunized subcutaneously once with H-2Db-restricted CTL peptides (RAH, 10 μg) mixed with or without 10 μg of murine CpG ODN (Invitrogen) in PELC (10%) adjuvant. After one week, splenocytes were harvested, and the RAH-specific CD8+ T cells were detected by tetramer staining using a PE-labeled RAH
tetramer (Beckman Coulter, CA, USA) and a FITC-labeled anti-CD8 mAb (eBioscience, CA, USA). The stained RAH-specific CD8+ T cells were analyzed by flow cytometry.

For the ELISPOT assay, spleens from the immunized mice were harvested and the response of IFN-γ-secreting cells was determined by ELISPOT after 48 h of peptide or protein re-stimulation. Briefly, 2 × 10^5 splenocytes were incubated with irrelevant peptides or RAH peptides in an anti-IFN-γ-coated PVDF plate for 48 h. After the incubation, the cells were removed, and a biotinylated anti-IFN-γ antibody (eBioscience, San Diego, CA, USA) was added to each well. The plates were then incubated at 37 °C for 2 h. Following the addition of the avidin-HRP reagent (eBioscience, CA, USA), the assay was developed with a 3-amine-9-ethyl carbazole (AEC; Sigma-Aldrich, MO, USA) staining solution. The reaction was stopped after 4–6 min by placing the plate under tap water. The spots were then counted using an ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH, USA).

2.6. In vivo cytolysis assay

The C57BL/6 mice were immunized subcutaneously once with RAH (10 μg) mixed with or without 10 μg of murine CpG ODN in a PELC (10%) adjuvant, and then after 7 days, CFSE-labeled target cells were adoptively transferred into the mice. The target cells were prepared from syngeneic splenocytes treated with a RBC lysis buffer (Biolegend) for 2 min to remove red blood cells. The cells were divided into two parts, one of which was pulsed with 5 μg/ml of RAH peptides for 30 min at 37 °C. After the splenocytes were washed in PBS, the cells only (non-peptide) and RAH peptide-pulsed cells were labeled at final concentrations of 1 μM CFSE (Molecular Probes, Eugene, OR) or 10 μM CFSE, respectively, for 15 min at 37 °C. The splenocytes were added to an ice-cold complete RPMI medium to stop the CFSE labeling. The cells only and RAH-pulsed splenocytes were re-suspended in PBS and mixed at a ratio of 1:1. The CFSE-labeled cells (2 × 10^7 per mouse) were adoptively transferred via tail vein injection into the immunized mice. The experimental cells were harvested 18 h after adoptive transfer and analyzed using FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Two equations were used to determine the percentage of lysis. The adjustment factor (A) was calculated by dividing the percentage of cells loaded without peptide by the percentage of cells loaded with RAH peptide in naive controls. For the percent lysis equation, the average of the As from 4 naive mice was calculated, and the following equation was used: % Specific lysis = ([% non-peptide × A] − [% RAH peptide]) / (% non-peptide × A).

2.7. Tumor regression study in mice

To assess the therapeutic value of the vaccines, tumors were first generated by injecting 2 × 10^5 TC-1 cells into the abdominal region of the mice, and seven days later, the mice were subcutaneously vaccinated in a separate abdominal region with the indicated dose of vaccine formulated with PELC and/or CpG. The tumor volume and tumor weight of the mice were measured on a daily basis for 21 days after vaccination. To evaluate whether this type of formulation allows the antigen or immunopotentiator to be controlled and slowly released, an in vitro release experiment was performed using RAH as a model peptide. The release of the PELC-formulated RAH determined in the mice relative to the induction of cell-mediated immunity. To evaluate whether this type of formulation allows the antigen or immunopotentiator to be controlled and slowly released, an in vitro release experiment was performed using RAH as a model peptide. The release of the PELC-formulated RAH determined in the mice relative to the induction of cell-mediated immunity. To evaluate whether this type of formulation allows the antigen or immunopotentiator to be controlled and slowly released, an in vitro release experiment was performed using RAH as a model peptide.

3. Results

3.1. The formulation of antigens with PELC

The preparation of the PELC-formulated nanoparticle as an adjuvant follows a two-step manufacturing process, as shown in Fig. 2A. In the first step, the aqueous solution of PEG-b-PLACL and the oil phase of squalene and Span®85 were pre-emulsified to form a stable and isotropic emulsion dubbed PELC. The aqueous solutions of antigen (RAH) and/or immunopotentiator (CpG) were mixed to form homogeneous particles in the second step. The size distribution of these particles was measured with a microscope and a particle size analyzer. Two different sizes were observed, relatively large particles of 1 μm and smaller ones of 200 nm (Fig. 2B). Notably, several studies have verified that small solutes or nanoparticles (<50 nm) are internalized by antigen-presenting cells (APCs) through macropinocytosis [34], whereas poly(lactide-co-glycolide) microparticles (>500 nm) and MF59 oil-in-water emulsion 200 nm in size can be internalized APCs through phagocytosis without specific recognition [34,35]. Here, PELC-formulated particles possess proper sizes for APCs uptake to facilitate the induction of cell-mediated immunity. To evaluate whether this type of formulation allows the antigen or immunopotentiator to be controlled and slowly released, an in vitro release experiment was performed using RAH as a model peptide. As we demonstrated that PELC nanoemulsion could prolong the release of antigen in vivo, the mice were immunized with various formulations that contains RAH-Alexa 647 and in-life fluorescence analysis was performed at 0, 6, 30, 48, 72, 144, 168, 240, 336 and 384 h. After the injection, the fluorescence signal was clearly presented on the dorsum of mice at 0 h in each group. After 72 h, the fluorescence signal dropped dramatically in both RAH-Alexa 647 alone (RAH group) and RAH-Alexa 647 mixed with CpG (RAH/CpG group) (Fig. 2A). Data analysis from ROI showed us that the fluorescence signals had about a 75–80% decrement at 30 h in the RAH and RAH/CpG groups (Fig. 2B). On the other hand, the significant amount of fluorescence signals were still visible in the PELC formulated groups (RAH/PELC and RAH/CpG/PELC) at 240 h (Fig. 2A, B). Both in vitro and in vivo results demonstrated that the PELC emulsified delivery system could serve as either carrier or vehicle to deliver biologically active agents (e.g., RAH peptide and CpG immunostimulatory adjuvants) to immune cells in a targeted and prolonged manner, thus effectively probing and manipulating the vaccine immunogenicity. These data were also confirmed in protein antigen (data not shown). Herein, PELC nanoemulsion has a depot effect that provides a sustained release profile of peptide/protein antigens.

3.2. PELC delay the release of antigen in vivo

To investigate the release rate of PELC-formulated peptide antigen in vivo, the mice were immunized with various formulations that contain RAH-Alexa 647 and in-life fluorescence analysis was performed at 0, 6, 30, 48, 72, 144, 168, 240, 336 and 384 h. After the injection, the fluorescence signal was clearly presented on the dorsum of mice at 0 h in each group. After 72 h, the fluorescence signal dropped dramatically in both RAH-Alexa 647 alone (RAH group) and RAH-Alexa 647 mixed with CpG (RAH/CpG group) (Fig. 2A). Data analysis from ROI showed us that the fluorescence signals had about a 75–80% decrement at 30 h in the RAH and RAH/CpG groups (Fig. 2B). On the other hand, the significant amount of fluorescence signals were still visible in the PELC formulated groups (RAH/PELC and RAH/CpG/PELC) at 240 h (Fig. 2A, B). Both in vitro and in vivo results demonstrated that the PELC emulsified delivery system could serve as either carrier or vehicle to deliver biologically active agents (e.g., RAH peptide and CpG immunostimulatory adjuvants) to immune cells in a targeted and prolonged manner, thus effectively probing and manipulating the vaccine immunogenicity. These data were also confirmed in protein antigen (data not shown). Herein, PELC nanoemulsion has a depot effect that provides a sustained release profile of peptide/protein antigens.

3.3. The RAH peptide formulated with PELC and/or CpG enhances specific CD8+ T cells

As we demonstrated that PELC nanoemulsion could prolong the release of antigens, we will identify the immune response of antigens after formulation with PELC and combination with or without CpG. The immunogenicity of the RAH peptide antigen after formulation with PELC, CpG or PELC/CpG was determined in the mice relative to the induction of the tumor antigen-specific CD8+ T cell response. The RAH peptide
was formulated with various emulsion formulae or PBS and injected subcutaneously into the C57BL/6 mice. After 7 days, splenocytes were isolated from the immunized mice and re-stimulated in vitro in the presence of RAH. Among the various formulations, we found that RAH formulated with PELC/CpG (RAH/PELC/CpG) induced the highest number of RAH-specific CD8+ T cells (0.32%) after immunization (Fig. 3A). However, there was no significant induction of RAH-specific CD8+ T cells in the immunization with the RAH peptide formulated with PELC or CpG alone compared to the RAH in PBS formulation (Fig. 3A). To further investigate the RAH-specific IFN-γ-secreting cells after immunization, the splenocytes from vaccinated mice were re-stimulated with RAH. We found that the number of specific IFN-γ-secreting cells was increased by immunization with RAH/PELC/CpG compared with RAH, RAH/PELC or RAH/CpG (Fig. 3B). Immunization with either RAH/PELC or RAH/CpG also resulted in slightly increased IFN-γ-secreting cells compared with RAH alone.

3.4. The RAH peptide formulated with PELC and CpG elicits a CTL response

We additionally sought to study the efficiency of different formulations for inducing a specific CTL response. To this end, the CTL responses were detected using the in vivo CTL assay. Briefly, the mice were immunized with RAH mixed with or without CpG in a PELC adjuvant. Seven days later, the immunized mice were adoptively transferred with CFSE-labeled target cells for 18 h. The purpose of this assay is to evaluate whether immunized mice can kill peptide-pulsed cells specifically. Unvaccinated mice showed similar amounts of specific lysis as mice vaccinated with RAH peptides alone; RAH/PELC-immunized mice exhibited a mildly increased killing activity. In the presence of CpG ODN, the RAH peptide or RAH/PELC could induce strong cytotoxic effects (Fig. 4A). The percentages of specific lysis were 0.52, 0.26, 0.90, 14.69, and 64.84% in the Control, RAH, RAH/PELC, RAH/CpG, and RAH/PELC/CpG groups, respectively (Fig. 4B). This result indicates that the PELC adjuvant combined with CpG synergistically elicits a CTL response when used in a peptide vaccine.

3.5. The RAH peptide formulated with PELC and/or CpG induces an anti-tumor effect

To determine whether the higher CTL response reflected anti-tumor activity in vivo, different RAH formulations were injected once 7 days after tumor inoculation. We found that immunization with RAH/PELC or RAH/CpG delayed the tumor growth, but the tumor totally regressed in the group that had been immunized with RAH/PELC/CpG (Fig. 5A). The mice in the PBS control or RAH alone groups all died before day 50 after tumor implantation. In contrast, the tumor-free survival rates were 30%, 50% and 100% for the RAH/PELC, RAH/CpG and RAH/PELC/CpG group, respectively (Fig. 5B). To extend the application of this formulation, recombinant mutant E7 protein (rE7m) was formulated with PELC/CpG (rE7m/PELC/CpG) to immunize the mice once. Significant CD8+ T cell response and tumor regression were observed after administering rE7m/PELC/CpG compared to rE7m/PELC or rE7m/CpG (Supplemental Fig. 1). Taken together, PELC combined with CpG adjuvant in a peptide/protein vaccine provides a robust CTL response and anti-tumor activity.

Fig. 1. PELC-formulated peptide/protein vaccine. (A) The schematic representation of the PELC-formulated peptide/protein vaccine preparation. The preparation of PELC-adjuvanted peptide vaccine follows a two-step procedure that includes emulsification and dispersion. First, an aqueous solution containing PEG-b-PLACL and an oily phase consisting of squalene and Span®85 were homogenized to form a stable and isotropic emulsion, PELC. Next, the pre-emulsified stock was dispersed into a peptide/protein solution to form homogeneous nanoparticles in the second step. (B) Homogeneous fine particles were observed with an Olympus DP70 microscope and laser light scattering analysis. (C) Cumulative release of RAH peptide from the PELC formulation. RAH was formulated with or without 10% PELC (50 μg per 50 μl) and placed in a dialysis chamber in a eppendorf containing 1 ml ddH2O. RAH release was monitored by the MicroBCA method. The data are presented as the mean ± SD of four samples.
4. Discussion

The use of synthetic peptides and proteins as vaccines is safe, but because of poor immunogenicity, adjuvants are critical for inducing protective immunity. Based on their effective mechanisms, adjuvants can be divided into two types—antigen delivery systems and immuno-potentiators. The induction of CTL responses using a peptide immunogen is not easy in the absence of adjuvants. To induce CTLs for killing cancer cells, the use of both an antigen delivery system and an immunopotentiator might generate synergistic effects. In the present study, we showed that using a synthetic peptide derived from HPV16 E7 or the recombinant mutant E7 (rE7m) as the immunogen could induce a significant anti-tumor effect in the presence of PELC and CpG. These new formulations could be potentially therapeutic vaccines for HPV-associated cancers.

4.1. The development of a W/O/W emulsion-type adjuvant (PELC) for cancer immunotherapy

In theory, good antigen delivery systems are able to target antigen-presenting cells, such as dendritic cells, efficiently or prolong antigen presentation by preventing degradation. Antigen delivery systems include emulsion-type adjuvants and particulate-type adjuvants. An oil-in-water (O/W) emulsion-type adjuvant, MF59, has been used in flu vaccines, which has been shown to enhance antibody titers in an elderly population [36,37]. MF59 has the advantages of low oil content and high injectability when performing vaccination. Regarding the mechanisms of adjuvant action, MF59 possesses high efficiency to the induction of an early and strong cytokine- and chemokine-rich environment at the site of injection, and the beneficial of modulation of genes involved in leukocyte migration and antigen presentation [38]. The E7 protein formulated in MF59 can generate CTL responses against E7-expressing cells, but the anti-tumor effect in vivo has not been reported [39]. In addition, the combination of polylactide co-glycolide polymer (PLG) microparticles and MF59 induced CTL activity against HIV-1 p24 gag [40]. However, further studies of MF59 as an adjuvant for cancer immunotherapy are limited. In contrast, the water-in-oil (W/O) emulsion-type adjuvant possesses dispersed antigenic media and continuous oily phases. This type of adjuvant has been evaluated to ascertain the innocuity of the vaccine and to achieve long-term protective immune responses. However, conventional incomplete Freund’s adjuvant (IFA) generates long-lived depots in the injection site that may lead to the trapping of tumor-specific CD8+ T cells at the injection site instead of promoting an effective T cell response at the tumor site as described in the literature [41]. The new generation of W/O adjuvant, Montanide™ ISA51 and ISA 720, has been used in clinical studies of cancer immunotherapy. The long peptides (23–45 amino acids) of HPV16 E6 and E7 formulated with ISA-51
induced strong T cell responses in a phase I clinical trial [42]. In a further evaluation of the therapeutic efficacy of this formulation, HPV16-positive women with high-grade vulvar intraepithelial neoplasia were evaluated. Nine of twenty vaccinated patients showed complete regression of all lesions and eradication of the virus [43,44]. These results showed that a peptide vaccine formulated with an emulsion-type adjuvant could generate strong T cell responses against an intraepithelial neoplasm. In the present study, PELC is a water-in-oil-in-water (W/O/W) emulsion-type adjuvant that is generally recognized as a more stable formulation [19]. It is probable that W/O/W not only reserved the depot effects intrinsic to W/O but also combined the antigen presentation effects belonging to O/W. Another advantage of the PELC formulation is that the pre-made emulsified PELC can simply be mixed with antigen before injection. This approach could reduce the potential for the incomplete emulsification of the vaccine because of the variability in individual compliance with procedures. Except for emulsion-type adjuvants, the peptides or proteins of HPV16 E7 have been incorporated into cationic liposomes, acid-degradable hydrogel particles [45] or virosomes [46] to treat established tumor models and have been shown to induce strong anti-tumor immunity [47–49]. These data demonstrated that an antigen delivery system adjuvant could be used to treat cancer. Here, our report showed that an easy-to-prepare PELC emulsion that was formulated with peptide enhanced the therapeutic effects of a cancer vaccine; in the presence of the TLR9 agonist (CpG) and PELC, the peptide formulation synergistically elicited anti-tumor immunity.

4.2. The incorporation of a TLR ligand in the PELC formulation enhanced anti-tumor activity

In addition to the delivery systems used in a vaccine adjuvant, the use of immunopotentiators as adjuvants also plays an important role in increasing the vaccine efficacy of immunogens, especially for peptides with poor immunogenicity. Immunopotentiators include cytokines (IL-2, GM-CSF) [50], chemokines (CCL5) [51], and innate receptor ligands (TLR2, TLR4 and TLR9) [52–54]. Because the innate receptors recognize conserved molecules expressed by a wide variety of infectious agents, using innate receptor ligands as adjuvants may mimic the natural infection of microbes. Currently, innate receptor ligands are recognized to be the new generation of adjuvants [55]. The TLR2 ligand, bacterial lipopeptide, has been used to manufacture a Lyme disease vaccine (LYMErix™) [56] and was licensed by the FDA in 1998 for general use. The monophosphoryl lipid A (MPL) is a TLR4 ligand that has been combined with aluminum salt (known as AS04) for hepatitis B vaccine (FENDrix®) or HPV vaccine (Cervarix®) [57]. Most recently, the unmethylated CpG motif in bacterial DNA has been identified as a TLR9 ligand that stimulates endosomal TLR9 in antigen-presenting cells (i.e., DC or macrophage). The synthetic oligodeoxynucleotide (ODN) contains unmethylated cytosine–guanosine motifs (CpG) that are usually composed of a phosphorothioate nucleotide. The preclinical studies indicated that CpG has a good safety profile and improves the activity of vaccines targeting infectious diseases and cancer [58–64]. The
Pan-DR Th epitope has been conjugated to a peptide of HPV16 E7 and formulated in a liposome and ISA-51 (called VaccMax®) with CpG and was shown to eradicate established tumors in vivo [65]. The combination therapy with cholera toxin-E7 fusion protein-pulsed DCs and the local treatment of the tumor with CpG was required for the complete eradication of cancer [66]. A new, low-dose, chemically stabilized form of the E7S0s (E7SOx) that has been combined with CpG as an adjuvant elicits a strong long-lasting protection against E7-expressed cancer [67]. Mansilla et al. reported that intravenous injection with the extracellular domain of fibronectin fused with E7, in combination with the TLR3 ligand, polyinosinic–polycytidylic acid (pIC), or CpG complexed with cationic lipids, was able to eradicate large established TC-1 tumors [68]. These promising results indicate that immunopotentiator CpG could be very useful as an adjuvant in cervical cancer immunotherapy.

5. Conclusion

Our work presented here demonstrated that immunization of the mice with the RAH peptide formulated with PELC or CpG alone induced specific CD8+ T cell responses and an anti-tumor effect. However, immunization with RAH/PELC mildly influenced the killing activity, presumably due to the difference in the induction times of the CD8+ T cell responses between immunization with RAH/PELC and RAH/CpG. Moreover, PELC-formulated peptide vaccine in the presence of CpG synergistically elicited a CTL response and inhibited tumor growth. This formulation may attenuate peptide degradation to protect the antigen and increase the half-life of the antigen. Taken together, we found that a peptide/protein antigen formulated with the novel emulsion-type adjuvant combined with an immune potentiate (TLR9 ligand) could induce strong CTL responses against cancer. The combination of an antigen delivery system and immunopotentiators is a superb strategy for the development of therapeutic tumor vaccines.

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