

Full-length article

Cytotoxic T lymphocyte response induced by an improved synthetic lipopeptide vaccine against cervical cancer

Dao-hua XU^{1,5}, Chen-hui ZHOU², Yong-peng XIA³, Zong-ying QIU³, Yu-zhang WU⁴, Zheng-cai JIA⁴, Wei ZHOU⁴

¹Department of Pharmacology and ²School of Nursing, Guangdong Medical College, Zhanjiang 524023, China; ³Department of Pharmacy, Chongqing University of Medical Science, Chongqing 400016, China; ⁴Institute of Immunology, PLA, Third Military Medical University, Chongqing 400038, China

Key words

epitope; cervical cancer; lipopeptide; vaccine

⁵ Correspondence to Dr Dao-hua XU.

Phn 86-759-238-8588.

Fax 86-759-223-4434.

E-mail daohuax@yahoo.com.cn

Received 2006-09-30

Accepted 2006-11-22

doi: 10.1111/j.1745-7254.2007.00538.x

Abstract

Aim: To explore cytotoxic T lymphocyte (CTL) response induced by the lipopeptide vaccine against cervical cancer. **Methods:** The immunological effect inducing CD8⁺ T cell-mediated cytotoxicity was investigated in human leukocyte antigen (HLA)-A2 transgenic mice and peripheral blood mononuclear cells (PBMC) of healthy HLA-A2.1+ blood donor. The activity of specific CTL was measured by using a standard 4 h ⁵¹Cr release assay. The content of major histocompatibility complex (MHC) I on T2 cells and the expression of immune molecules on dendritic cells (DC) were detected by flow cytometry, and the concentrations of interleukin (IL)-12 and interferon- γ were determined by ELISA. **Results:** The lipopeptide induced a strong epitope-specific CTL response both *in vivo* (transgenic mice) and *in vitro* (human PBMC). This CTL induction was critically dependent on the presence of the helper T lymphocyte epitope in transgenic mice, and the presence of a lipid tail bypassed the need for an adjuvant. The stability and persistence of the antigenic complex formed with the lipopeptide increased in comparison with the CTL parental peptide. The lipopeptide could induce the production of IL-12 in DC, but not the maturation of DC directly. **Conclusion:** The combination of CTL and the T helper epitope and lipid molecule can remarkably improve the immunogenicity of the CTL peptide, the mechanism of which is associated with an increase in the stability and persistence of the antigenic complex formed with the lipopeptide and in the production of IL-12 in DC induced by the lipopeptide. The lipopeptide can be considered a more effective vaccine type for human being.

Introduction

Cervical cancer is one of most common cancers in women. Human papillomaviruses (HPV), particularly HPV-16, are associated with most cervical cancers^[1,2]. HPV oncogenic proteins, E6 and E7, are expressed in most HPV-containing cervical cancers and are constitutively expressed in cervical carcinoma cells. They are the only viral proteins whose expression is required for the maintenance of the transformed state^[3-5]. Compared with E6, E7 has stronger immunogenicity^[6], therefore, E7 is an attractive target for the specific immunotherapy of cervical carcinoma.

Cytotoxic T lymphocyte (CTL) response is an important

defense mechanism against tumors^[7,8]. CTL recognize immunogenic peptides derived from cellular proteins presented to the cell surface in the content of MHC class I molecules. Many studies demonstrate that immunization with antigenic peptides alone is not sufficient to elicit an efficient CTL response^[9]. Studies have shown that helper T lymphocyte (Th) immunity is also considered vital for the CTL induction^[10-13]. Lipopeptide formulations have been successfully used to induce CTL responses and have recently gained considerable interest and are a promising vaccine candidate^[14-16]. Moreover, the peptide of the antigen HPV E7₈₆₋₉₃ is restricted to human leukocyte antigen (HLA)-A*2010, the most common HLA class I molecule, which makes it a good

candidate for vaccination^[17]. The TT₈₃₀₋₈₄₃ peptide, derived from tetanus toxoid, was chosen as a source of T cell help because of its promiscuous binding to human MHC class II antigens and its promiscuous recognition by T cells^[18]. A dipalmitic lipopeptide could induce epitope-specific CTL in phase I clinical studies^[19,20], but it is difficult to be synthesized and purified. So we constructed a monopalmitic lipopeptide (Table 1).

We evaluated the ability of the lipopeptide to provoke *in vivo*- and *in vitro*-specific CTL responses in HLA-A*0201K^b transgenic (Tg) mice and peripheral blood mononuclear cells (PBMC) preparations from MHC-matched healthy donors. We also studied the presentation of peptides in T2 cells and dendritic cell (DC) maturation induced by peptides.

Our findings show that the lipopeptide can induce a strong CTL response, the mechanisms of which are associated with an increase in the stability and persistence of the antigenic complex formed with the lipopeptide and in the production of interleukin (IL)-12 by DC. These findings suggest that the lipopeptide against cervical cancer can be considered as a more effective vaccine type for a human.

Materials and methods

Peptide synthesis and characterization The HLA-A*0201-restricted the CTL epitope derived from the HPV oncogenic protein, E7, referred to here as the P3 peptide, was used in this study (Table 1)^[21]. The lipopeptide construct is referred to here as the P1 lipopeptide. An unlipidated version denoted P2 was used as the control peptide (Table 1).

All peptides were synthesized on an ABI 431A instrument using standard Fmoc technology (PE Applied Biosystems, Foster city, CA, USA)^[22]. The solid support was Wang resin (Sigma, St Louis, MO, USA). The Fmoc protecting the group was removed by 20% piperidine in N-methylpyrrolidone (NMP). To synthesize the lipopeptide, a Boc-Lys-(Fmoc)-OH group was incorporated at the N-terminal extremity, which allowed the selective removal of the

Fmoc group by 20% piperidine in NMP. Then the cold peptide-resin was placed into the cold cleavage mixture (0.25 mL ethanedithiol, 0.25 mL water, 9.5 mL trifluoroacetic acid). The reaction mixture was stirred at room temperature for 1.5 h. Then these peptides were removed from the resin support and precipitated by adding cold diethyl ether and lyophilized. These crude peptides were purified by RP-HPLC. All peptides were checked for homogeneity by analytical RP-HPLC and for identity by an electrospray mass spectrometer. The purity was >85% for all peptides. The peptides were dissolved in DMSO at a concentration of 20 g/L and stored at -20°C.

Cells lines Human transporter associated with antigen processing (TAP)-deficient T2, cervical carcinoma Caski cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in culture according to the supplier's specifications. A stable transfectant of the jurkat human cell line that expresses the chimeric class I molecule, A*0201K^b, was kindly provided by Dr Jihad CHARO (Max Delbruck Center for Molecular Medicine, Berlin, Germany). The cell lines were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) with glutamax containing 10% fetal calf serum (FCS), antibiotics, N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), and 0.4 g/L G418 (Gibco, USA).

Animal immunization procedures and generation of CTL HLA-A*0201K^b Tg mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were bred and maintained in specific, pathogen-free facilities. Groups of 8-12 week-old HLA-A*0201K^b female Tg mice (5 mice per group) were immunized with synthetic peptide +/- incomplete Freund's adjuvant (IFA). Synthetic peptides were injected with 50 nmol per mice at the base of the tail for subcutaneous route. Transgenic mice were boosted 2 weeks later with the same synthetic peptide. After 14 d, the mice were killed and the spleens were aseptically removed. Splenic single cell suspensions were produced by teasing the organs through a sterile nylon mesh as previously described^[21]. The splenic suspensions (3×10^6) from the immunized mice were cocultured

Table 1. Structure of P1, P2, and P3.

Peptide	Lipid part	Adapter sequence	Th epitope sequence	Linker sequence	CTL epitope	Carboxyl terminus
P1	Palmitic acid	KSS	QYIKANSKFIGITE	GGG	TLGIVCPI	OH
P2	-	-	QYIKANSKFIGITE	GGG	TLGIVCPI	OH
P3	-	-	-	-	TLGIVCPI	OH

with 1 µg/mL P3 peptide in 5% CO₂ at 37 °C. Cytotoxic effector populations were harvested after 5 d of *in vitro* culture.

Generation of CTL in healthy donors The CTL induction *in vitro* was performed in accordance with the previously described procedures^[23,24]. In brief, the PBMC of 3 HLA-A*0201 healthy donors from the blood bank of the Third Military Medical University (Chongqing, China) were first obtained with centrifugation at a Ficoll-Paque density gradient and then cultured in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, and 0.1 g/L streptomycin. These cells were pulsed by the synthetic peptides, respectively, at a final concentration of 10 µmol/L. The PBMC were restimulated every 7 d with fresh medium containing these peptides. Recombinant human interleukin (IL)-2 (Sigma, USA) at a concentration of 30 IU/mL was added to the culture medium on d 1 after every stimulation. On d 6 after the third round of restimulation, the cells were harvested and tested by cytotoxicity assay.

Cytotoxicity assay The cytotoxic activity of the cell culture was determined by a standard 4 h chromium release assay^[25]. To measure the peptide-specific responses, T2 cells and jurkat A*0201K^b cells were loaded with 10 µmol/L peptide at 37 °C for 1 h. Peptide-pulsed T2 cells, jurkat A*0201K^b cells, and the Caski cell line were labeled with 3.7 MBq Na₂⁵¹CrO₄ for 1.5 h at 37 °C. Target cells (10⁴/well) were transferred into a round-bottomed, 96-well plate. Varying numbers of CTL were added to give a final volume of 200 µL and incubated for 4 h at 37 °C. At the end of the assay, the supernatant was harvested and counted. The percentage of specific lysis was calculated as follow: (experimental release - spontaneous release)/(maximal release - spontaneous release) × 100. Spontaneous and maximal release were determined in the presence of medium or 1% Triton X-100, respectively.

Cell isolation and generation of DC from PBMC The generation of DC from PBMC was performed as described previously^[26]. In brief, peripheral blood was collected from health volunteers (*n*=3) and fractionated over Ficoll-Paque by a standard procedure. To derive DC, PBMC were seeded (2 × 10⁶/mL) into 6-well plates in RPMI-1640 medium supplemented with 100 units/mL penicillin and 0.1 g/L streptomycin. After 2 h of incubation at 37 °C, nonadherent cells were removed and the adherent cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma, USA), 500 U/mL IL-4 (Sigma, USA), 100 U/mL penicillin, and 0.1 g/L streptomycin for 4 d at 37 °C. Immature DC were incubated *in vitro* for 48 h with P1, P2, or P3 peptides. Medium-treated, immature DC and tumor necrosis factor (TNF)-α-stimulated DC were included as negative and positive

controls, respectively. The phenotype of DC was analyzed by flow cytometry.

Interferon (IFN)-γ and IL-12 detection IFN-γ secretion in stimulated *in vitro* (IVS) culture supernatants and IL-12 in immature DC cultures were measured by a commercially available, two-site sandwich ELISA (Diaclone, Besancon, France) according to the manufacturer's instructions. Collection of the supernatant was performed at 72 h after IVS. DC were pulsed with P1, P2, or P3 peptides and incubated with autologous PBMC in RPMI 1640 medium. After 48 h of culture, IL-12 concentrations in the supernatant were determined. Recombinant IFN-γ and IL-12 were used for the preparation of a standard curve. Each sample was tested in duplicate.

Peptide-binding assay To determine whether synthetic peptides could bind to HLA-A*0201 molecules, peptide-induced HLA-A*0201 upregulation in T2 cells was examined according to a protocol described previously^[27]. Briefly, T2 cells were incubated with 10 µmol/L peptides and 5 µg/mL human β₂-microglobulin (Sigma, USA) in serum-free Iscove's Modified Dulbecco's medium for 18 h at 37 °C. The expression of HLA-A*0201 on T2 cells was then determined using anti-HLA-A*0201 mAb B B7.2 followed by a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Becton Dickinson, Mountain View, CA, USA). The samples were analyzed on a FACScan Calibur (Becton Dickinson, USA).

Stability of HLA-A*0201-peptide complexes on cell surface^[22] The T2 cells were incubated with 10 µmol/L peptides as described for the peptide-binding assay. After 18 h incubation at 37 °C, the T2 cells were washed. At the indicated times, they were stained with mAb B B7.2 and analyzed by flow cytometry.

Flow cytometry Standard flow cytometric analysis was used to assess surface expression of various markers using the following mAb directly conjugated with either phycoerythrin (PE) or FITC: FITC-CD40, FITC-CD80, FITC-CD83, and PE-HLA-DR (Pharmingen, San Diego, CA, USA). IgG isotype-matched control antibodies were used in all of the experiments. After staining, the cells were washed and fixed in 1% paraformaldehyde before analysis on a FACScan Calibur (Becton Dickinson, USA).

Statistical analysis The Student's *t*-test was performed to evaluate the significance of the results. *P* values less than or equal to 0.05 were considered significant.

Results

Immunological effect in transgenic mice after vaccination with peptides To assess their *in vivo* immunogenicity, HLA-A*0201K^b Tg mice were immunized with each of the

peptide in IFA. Splenocytes from immunized mice were restimulated *in vitro* and tested for IFN- γ production and CTL reactivity. CTL from P1-immunized mice showed strong IFN- γ production, an indicator of CTL reactivity. CTL from P1-immunized mice efficiently lysed jurkat A*0201K^b cells added with the E7₈₆₋₉₃ peptide, but did not lyse jurkat A*0201K^b cells alone. These results indicated that the cytotoxicity of induced CTL was peptide specific. CTL from P2-immunized mice also showed modest IFN- γ production and cytotoxicity. In contrast, immunization with the P3 peptide in IFA was shown to have no specific reactivity (Figure 1).

Induction of CTL by the lipopeptide without an adjuvant

To further explore the potential of the lipopeptide for human vaccination, we investigated whether the lipid part of the lipopeptide construct could bypass the requirement for IFA. Separate groups of Tg mice were immunized either with the lipopeptide or with an equimolar amount of the unlipidated version. Peptide-specific CTL responses were examined in the spleens of Tg mice. CTL from lipopeptide-immunized mice efficiently lysed jurkat A*0201K^b added with the E7₈₆₋₉₃ peptide, but not the unlipidated analogue P2. These data indicated that the lipopeptide could efficiently induce specific CTL responses *in vivo* without the need for an adjuvant (Figure 2).

In vitro generation of peptide-specific CTL from healthy human donors

To investigate the capacity of peptides to mobilize a human CTL repertoire, specific CTL were generated *in vitro* using healthy HLA-A*0201+donor PBMC with peptides. As shown in Figure 3, CTL induced with P1, P2, and P3 peptides lysed T2 cells pulsed with E7₈₆₋₉₃ and produced strong IFN- γ , but did not lyse T2 cells alone. Moreover, CTL induced by the P1 peptide elicited the strongest cytotoxic activity. To analyze the ability of specific CTL to lyse the endogenously HPV E7-expressing tumor cell, the HPV E7-positive HLA-A*0201-expressing cervical cancer cell line, Caski, was used as the target cell in a standard ⁵¹Cr-release assay. As shown in Figure 3, CTL were able to efficiently lyse Caski tumor cells (Figure 3).

Peptide-binding affinity to HLA-A*0201 molecules

Peptide-binding affinity to HLA-A*0201 molecules was analyzed by a binding assay on the TAP-deficient, human T2 cell line. The stabilization assay was based on the fact that in T2 cells, a large proportion of MHC class I molecules are devoid of endogenous peptides and are unstable at 37 °C. This could be prevented by the addition of exogenous peptides that bind within the MHC class I Ag-presenting groove, stabilizing the 3-D structure of the complex. According to our results, stabilization of MHC class I on T2 cells could be obtained with the lipopeptide (Figure 4).

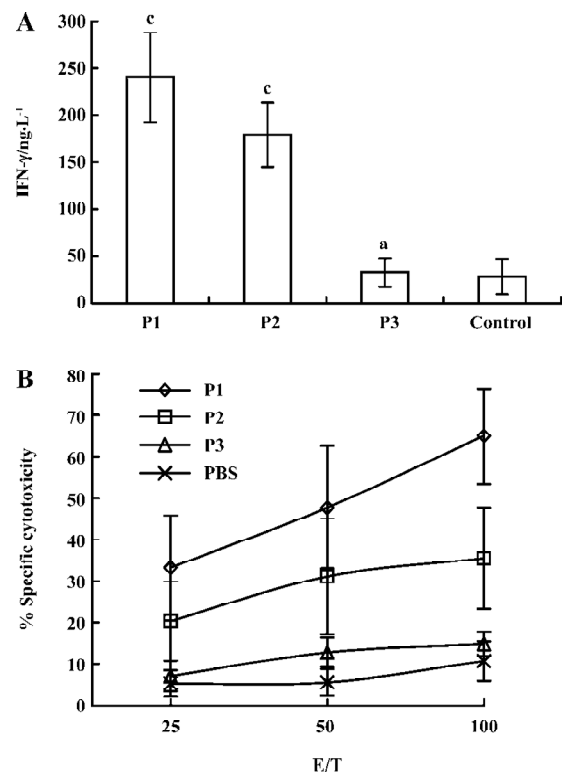


Figure 1. Specific CTL response of HLA-A*0201K^b-transgenic mice immunized with P1, P2, or P3 in IFA. IFN- γ released by bulk CTL from immunized mice was detected by ELISA assay (A) and lytic activity was tested using a standard 4 h chromium release assay (B). Mice were immunized subcutaneously at the base of the tail with peptides in IFA with a booster 2 weeks later. After 2 weeks, the spleens were harvested, and *in vitro* stimulation (IVS) were done. (A) IFN- γ was performed at 72 h after IVS; (B) targets were jurkat A*0201K^b loaded with peptide (E7₈₆₋₉₃). n=5. Mean \pm SD. ^aP>0.05, ^cP<0.01 vs control group.

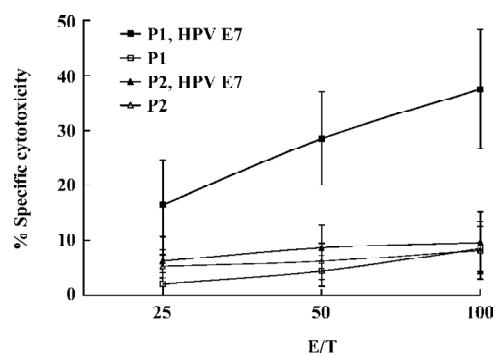


Figure 2. Specific CTL induction by the lipopeptide in HLA-A*0201K^b-transgenic mice without IFA. Mice were immunized subcutaneously at the base of tail with the lipopeptide or an unlipidated version and no additional adjuvant, with a booster 2 weeks later. After 2 weeks, the spleens were harvested, and IVS were done. Targets were jurkat A*0201K^b loaded with peptides (E7₈₆₋₉₃, filled symbols) and without peptides (open symbols). n=5. Mean \pm SD.

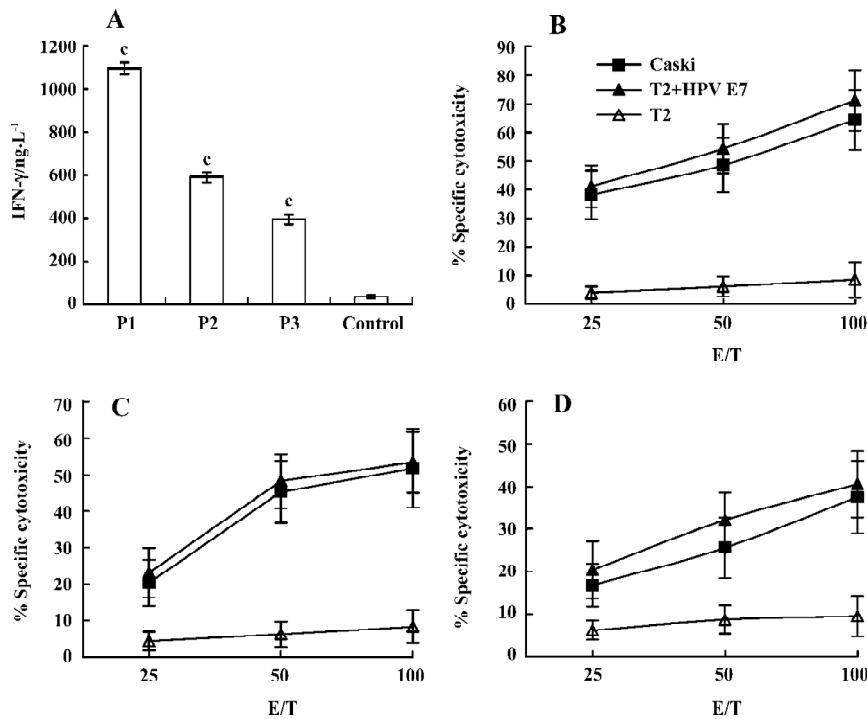


Figure 3. Specific reactivity of human CTL induced by P1, P2, and P3 peptides, respectively. IFN- γ release by bulk CTL from PBMC was detected by ELISA assay (A), and lytic activity was tested using a standard 4 h chromium release assay (B, C, D). Effectors were induced from PBMC with the synthetic peptide (B) P1, (C) P2, (D) P3. $n=3$. Mean \pm SD. $^cP<0.01$ vs control group.

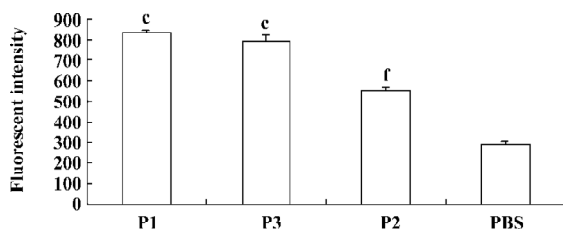


Figure 4. HLA-A*0201-binding affinity of peptides. Experiment was performed with T2 cells. After the peptides were incubated for 18 h at 37 °C in medium supplemented with β 2-microglobulin, the cells were stained with anti-HLA-A*0201 followed by detection with FITC-labeled goat anti-mouse IgG. Cells were analyzed by flow cytometry. $n=3$. Mean \pm SD. $^cP<0.01$ vs P2 group. $^fP<0.01$ vs phosphate buffer solution (PBS) group.

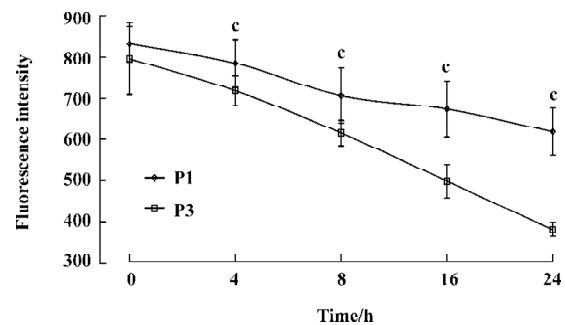


Figure 5. Complex stability assay. Experiment were performed with T2 cells. After the peptides were incubated for 18 h at 37 °C in medium supplemented with β 2-microglobulin, the T2 cells were washed. At the indicated times, the cells were stained with anti-HLA-A*0201 followed by detection with FITC-labeled goat anti-mouse IgG. Cells were analyzed by flow cytometry. $n=3$. Mean \pm SD. $^cP<0.01$ vs P3 group.

Complex stability assay The kinetics of complex dissociation was evaluated by measuring the time necessary for the complex to disappear from the cell surface. Our observations revealed an increase in the persistence of the antigenic complex formed with the P1 peptide (Figure 5).

DC maturation induced by peptides To check whether the addition of peptides induce DC maturation, the DC were incubated further for 48 h with or without peptides. TNF- α was used as a maturation control. The mean surface fluorescence intensity of molecules involved in the antigen presentation and in costimulation was not increased, and CD83 expression was not induced by the peptides (Figure 6).

IL-12 secretion of DC induced by peptides To check whether the exposure of DC to peptides increases IL-12 secretion, the DC were pulsed with P1, P2, or P3 peptides and incubated with autologous PBMC in RPMI 1640 medium. After 48 h of culture, IL-12 concentrations in the supernatant were determined. There was an upregulation of production of IL-12 following incubation of DC with P1, P2, or P3 peptides, and the effect of the P1 peptide was the strongest (Figure 7).

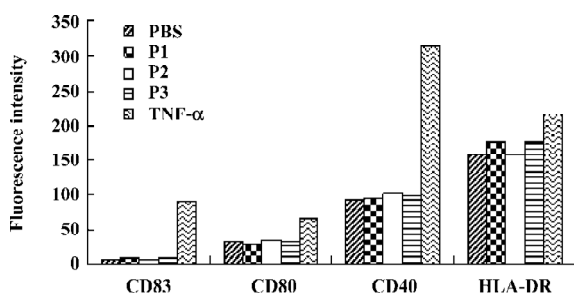


Figure 6. DC maturation induced by P1, P2, or P3 peptide. Immature DC were incubated alone or in the presence of P1, P2, P3 peptide, or TNF- α for 2 d. Surface molecules were labeled and analyzed by flow cytometry. Experiment was representative of 2 independent experiments.

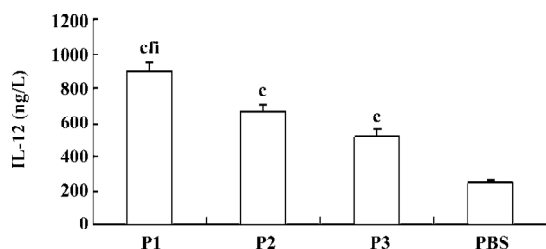


Figure 7. IL-12 secretion of DC induced by peptides. DC were pulsed with P1, P2, or P3 peptides and incubated with autologous PBMC in RPMI-1640 medium. After 48 h of culture, IL-12 concentrations in the supernatant were determined. $n=3$. Mean \pm SD. $^cP<0.01$ vs PBS group. $^{cfi}P<0.01$ vs P3 group. $^iP<0.01$ vs P2 group.

Discussion

Traditional immunization includes vaccines that use live attenuated organisms, inactivated organisms, whole proteins, and naked DNA. From a practical and safe standpoint, however, live, attenuated vaccines raise issues related to manufacturing and safety that may preclude their widespread use. Peptide-based approaches offer several potential advantages over conventional whole proteins or naked DNA in terms of purity, lot-to-lot consistency, cost of production, and a high specificity in eliciting immune response^[15,28–31]. This technology has proven to be an extremely useful strategy to avoid potentially harmful immune responses. However, immunization with antigenic peptides alone is not sufficient to elicit an efficient CTL response^[9]. It is important for peptide-based vaccine development to improve the immunogenicity of peptides.

Our study showed P2 containing Th and CTL epitopes could induce CTL response, but P3 only containing the CTL epitope could not do so in Tg mice. The result showed the

necessity for the help effect of Th cells in the induction of CTL, which was in agreement with recent studies^[12,32]. Our study also showed that P1 containing Th, CTL, and lipid part had already induced the strongest CTL response, which showed that linkage of Th, the CTL epitope, and lipid part could improve the immunogenicity of the CTL epitope. Of particular interest was the finding that peptide-specific CTL were able to lyse Caski tumor cells, which increased the possible clinical use of the lipopeptide as a cancer vaccine.

We investigated whether the lipopeptide could bypass the need for IFA. Our study confirmed that a lipopeptide alone could induce CTL response, and demonstrated that such an immunogen bypassed the requirement for an adjuvant. The toxicity of a lipopeptide was low and a patient could easily tolerate the toxicity, so the lipopeptide might be a good vaccine for human use^[15,16,33].

Loing and colleagues reported that the lipopeptide could gain access into the MHC-loading compartment of T2 cells without the help of the TAP transport system, and the TAP-deficient T2 cells could specifically present the lipopeptide to CD8+ T cells^[22]. Our data indicated that the lipopeptide could be processed and presented by T2 cells. Moreover, studies have shown that the lipopeptide could also be presented by powerful antigen-presenting cells (APC)^[34,35], which provide an additional advantage to the lipopeptide construct in terms of immunogenicity potential.

IL-12 forms a link between innate resistance and adaptive immunity and plays an essential role in antitumor immune response. IL-12 has been shown to favor a Th1 response and to enhance the generation of CTL^[36]. Our results showed that the peptide could induce the production of IL-12 of DC, which was one of the mechanisms of the lipopeptide enhancing the immunogenicity of CTL epitope peptides.

DC are the only APC capable of activating naive lymphocytes, resulting in the initiation of protective immune responses. In the absence of strong DC activation, naive T cell will differ and die. Only properly matured DC will deliver the signals required for full memory and/or effector CTL induction. In other words, immature DC tolerate while mature DC immunize^[37,38]. Our results showed that the lipopeptide could not directly induce the maturation of DC, but the lipopeptides could activate Th and enhance the expression of the CD40 ligand (CD40L) on Th. Then CD40L on Th interacted with CD40 on DC, by which DC was activated^[39].

In our observation, the binding affinity of peptide vaccines to the HLA molecules and their stability, as well as levels of IL-12 production from DC by peptide vaccines

appear to be important for induction of CTL responses. In our study, however, P3 peptides showed a higher binding affinity to HLA molecules, as compared to P2 peptides. In contrast, P2 peptides displayed a higher CTL activity than P3 peptides. This discrepancy in the importance of the peptide binding affinity vs CTL induction might be explained by the possibility that P2 peptides (Th plus CTL epitopes) might get a help from Th cells for improved CTL induction, as compared to P3 peptides (CTL epitope alone).

In conclusion, the lipopeptide can induce a strong CTL response, the mechanisms of which are associated with an increase in the stability and persistence of the antigenic complex formed with the P1 peptide and in the production of IL-12 of DC. These findings suggest that the lipopeptide against cervical cancer can be considered a more effective vaccine type for a human. Moreover, the lipopeptide can be synthesized in pure form, providing the ability to develop rapidly this type of vaccine at a low cost.

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