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Enhancement of Immunotherapeutic Effects of HPV16E7 on Cervical Cancer by Fusion with CTLA4 Extracellular Region

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Cervical cancer is caused by infection by high-risk human papillomavirus (HPV), especially HPV16. Limitations in current treatments of cervical cancers call for the development of new and improved immunotherapies. This study aims at investigating the efficacy of a novel vaccine consisting of modified HPV 16E7 fused with human cytotoxic T-lymphocyte antigen 4 (CTLA4). The regions in HPV16 E7 gene associated with its transformation and CTL-enhanced response were modified; the resultant HPV16mE7 was fused with extracellular region of CTLA4 to generate HPVm16E7-eCTLA4 fusion protein. Binding of this fusion protein to B7 molecules expressed on antigen presenting-cells (APCs) was demonstrated. C57BL/6 (H-2^b) mice immunized with low dose of the fusion protein (10 μg) produced higher titer antibody and stronger specific CTL response, and expressed higher levels of IFN-γ and IL-12, compared with those immunized with HPVm16E7 only or admixture of HPVm16E7 and CTLA4, or PBS; and were protected from lethal dose tumor challenge. Tumor growth was retarded and survival prolonged in mouse models with the fusion protein treatment. Our results demonstrate that fusion of HPV16 E7 with eCTLA4 targeting APCs resulted in enhanced immunity, and that this fusion protein may be useful for improving the efficacy of immunotherapeutic treatments of cervical cancer and other HPV16 infection-associated tumors.

***Keywords*:** cervical cancer, human papillomavirus 16 (HPV16), cytotoxic T-lymphocyte antigen 4 (CTLA4), vaccine

Cervical cancer (CC) causes about 280,000 deaths annually worldwide, with women in developing countries accounting for 80% of these deaths (WHO, 2004). There is now compelling evidence, including clinical, molecular, and epidemiological investigations, that infection of the cervical epithelium with HPV increases the risk of premalignant lesions and progress to cervical cancer, with high risk types HPV-16 and 18 accounting for nearly 70% of cervical cancer cases (Berzofsky *et al*., 2004; Mahdavi and Monk, 2005; Kanodia *et al*., 2008; Psyrri and Daniel, 2008). Integration of the viral genome into the host cell genome, a necessary step in cellular transformation, leads to the loss of some genes necessary for a productive viral life cycle and to deregulated or over expression of the E6 and E7 oncoproteins which interact with p53 and retinoblastoma tumor suppressor protein (pRB), respectively (Dyson *et al.*, 1989; Werness *et al*., 1990). Current treatment of cervical cancer is limited to excision or ablative procedures that remove or destroy cervical tissue. These procedures have efficacy rates of approximately 90% but are associated with morbidity and expense. Additionally, surgical treatments remove only the dysplasia tissue, leaving normal appearing HPV-infected tissue untreated (Bell and Alvarez, 2005). This situation creates needs and opportunities to develop novel and improved therapeutic approaches for cervical cancers and other HPV infection-associated malignancies.

Therapeutic vaccines present far more challenges than prophylactic vaccines. The challenges include the immune-compromised status of cancer patients, difficulty in stimulating the immune system, immune escape mechanisms used by tumors and virally infected cells (Zur Hausen 2002; Berzofsky *et al.*, 2004). Unlike most tumor-specific antigens derived from normal or mutated endogenous proteins, HPVE6 and E7 are completely foreign viral proteins, and thus may harbor more antigenic peptide/epitopes than a mutant cellular protein. Because E6 and E7 are required for induction and maintenance of the malignant phenotype of cancer cells, cervical cancer cells are unlikely to evade an immune response through antigen loss. Thus, various forms of HPV vaccines targeting HPV E6 and E7 proteins have been described in experimental systems, including plasmid DNA, viral or bacterial vectors, chimeric virus-like particles, synthetic peptides, and recombinant proteins (Mahdavi and Monk, 2005). Some of these approaches that were safe enough for testing in humans were brought to the clinic where their safety profiles were confirmed along with their potency to induce specific cellular immunity in phase I/II studies, albeit with limited clinical support (Mahdavi and Monk, 2005). Yet, it remains of great significance and interest to evaluate novel tools to target HPV to the immune system for induction of cell-mediated responses. Due to many tumor antigens alone being not enough to induce strong cellular immunity, therapeutic vaccines may be chemically

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coupled to a strongly immunogenic carrier protein and mixed with an adjuvant to induce a stronger immune response. Alternatively, genetic fusion of the oncoprotein with various cytokine molecules, including interleukin (IL)-2, IL-4, and granulocytemacrophage colony-stimulating factor (GM-CSF) converts this self tumor antigen (Ag) into a strong immunogen capable of inducing protective immunities without carrier proteins or adjuvants (Tao and Levy, 1993; Chen *et al*., 1994).

The orchestration of effective T-cell immune responses depends not only on antigenic stimuli but also on a plethora of cell surface proteins (costimulatory molecules) expressed on T cells and APCs capable of amplifying T-cell activation. Among many costimulatory molecules, CD28 has a primary role in the activation of T cells by signaling through the costimulation pathway, which is dependent on its binding to B7 molecules expressed on APCs. On the other hand, CTLA4, a protein expressed on activated T cells, plays a negative role in dampening the response by binding to the set of B7 molecules. CTLA4 can form a homodimer, but dimerization of CTLA4 is not required for B7 binding. Importantly, CTLA4 binds B7 proteins more avidly than CD28 and this property was exploited to design immunomodulatory reagents (e.g., CTLA4-Ig) (Linsley *et al.*, 1994; Jeffrey *et al*., 2006), which served as valuable tools in a number of immunotherapeutic settings.

In several studies, DNA vaccines harnessing fusion CTLA4 to antigen were exploited for antitumor and antivirus purposes (Boyle *et al*., 1998; Deliyannis *et al.,* 2000; Nayak *et al.*, 2003; Tian *et al.*, 2004). Our present study sought to assess the efficacy of a novel fusion protein consisting of modified HPV16E7 and the extracellular region of human CTLA4 in the immunotherapy of tumor models expressing HPV16E6E7 protein.

Materials and Methods

HPV16 E7 gene and mutagenesis and peptides

HPV16 genomic DNA was extracted from the epithelial cells of uterus cervix of young females with HPV infection. HPV16E7 was amplified by PCR and sequenced. The 98-residue native HPV16E7 protein contains three regions that are arbitrarily designated as (A), (B), or (C) (Fig. 1). In the (A) region, amino acids 21, 24, and 26 were mutated from DLYCYEQ to GLYGYGQ to reduce transformation activity and enhance antigenicity (Munger, *et al*., 2001; Cassetti *et al.*, 2004; Joeli *et al.*, 2007). In the (C) region two zinc finger binding motifs were disrupted by modifications of C61 to G61 and C94 to G94 (Fig. 1) using the mutagenesis kit from TaKaRa (USA).

The synthetic peptides E7.49-57 (RAHYNIVTF) corresponding to the HPV16-E7 H2-Db-restricted epitope (Feltkamp *et al.*, 1993) and HBV surface antigen (HBsAg) R187 (aa183-191) (FLLTRILTI) (Liu *et al*., 2008) were purchased from Sangon (China).

Construction of plasmids

Modified HPV16E7 (designated as HPV16mE7) gene was cloned into pET-28a vector (Novagen). The human CTLA4 gene was from plasmid pCDNA-hCTLA4 (gifted by Prof. Tao, the Institute of Biomedical Sciences, Academia Sinica, Taipei). To express human CTLA4 protein, CTLA4 gene was cloned into pET-his2.9 plasmid. To construct the 16mE7-eCTLA4 fusion protein, we used overlap polymerase chain reaction (PCR) to replace the signal sequence of the human CTLA4 gene with a sequence encoding the GGGGSGGGGS (Gly4Ser) 2 peptide linker and introduced a stop codon upstream of the transmembrane domain. The forward primer 5'-GGAGGCGGGGGCTCGATGCACGTGGCCCAGCCTG-3' and the reverse primer 5'-GGATC*GCTAGC*TCAGTCAGAATCTGGGCACGGTTC-3' (*NheI*) were used in the first round PCR on the human CTLA4 gene. The resulting PCR product was used as template to perform a second round PCR using the same reverse primer and the 3' end of the second forward primer and a second forward primer 5'-GAATTCAAGCTTGGAGGCGGGGGCTCGGGAGGCGGGGGCTCG-3'. The 5' end of the first forward primer and the 3' end of the second forward primer have a 15 nucleotide overlap. The final PCR product was ligated to generate pET-16mE7-eCTLA4, which was verified by sequencing.

Expression and purification of HPV16mE7, CTLA4 and 16mE7-eCTLA4 proteins

The verified plasmids were then transformed into the *E. coli* strain BL21γDE3 (Novagen). Recombinant proteins containing His-Tag were expressed upon induction with 0.5 mmol/L isopropyl-ß-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, USA) and purified on Ni-NTA agarose (QIAGEN, Germany). Isopropanol washes were applied to remove lipopolysaccharide contamination. Purified proteins were dialyzed and lyopholized.

Western blot analysis for HPV16mE7, CTLA4, and 16mE7-eCTLA4

Proteins were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (Bio-Rad, USA) which was probed with either a mouse anti-HPV16-E7 monoclonal antibody (Santa Cruz Biotechnology, USA) or a mouse anti-His antibody (Santa Cruz Biotechnology, USA). Immune complexes were detected with goat anti-mouse immunoglobulins conjugated to HRP (Chemicon, USA).

Immunofluorescence and FACS analysis of HPV16mE7-eCTLA4 binding to B7 expressed on dendritic cells

Mouse bone marrow (BM)-derived dendritic cells (DCs) were prepared as described (Kevin *et al*., 2006). Briefly, mouse BM was flushed from the hind limbs, passed through a nylon mesh, and depleted of red cells with ammonium chloride. After extensive washing with RPMI-1640, cells were cultured with RPMI-1640 supplemented with 10% FBS, recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) and recombinant mouse interleukin-4 (IL-4) (20 ng/ml) (both from PeproTech, USA). On days 2 and 4 of culture, the supernatant was removed and replaced with fresh medium containing mGM-CSF and mIL-4. All cultures were incubated at 37°C in 5% humidified CO_2. Non-adherent granulocytes were removed after 48 h of culture, and fresh medium was added. After 7 days of culture, more than 80% of the cells expressed characteristic DC-specific markers as determined by fluorescence-activated

cell sorting (FACS). DC cells were fixed and permeabilized with methanol: acetone (1:1, v/v) for 3 min, incubated with purified and diluted HPV16mE7, human CTLA4, and HPV16mE7-eCTLA4 at RT for 1 h, and washed three times for 5 min with phosphate-buffered saline (PBS). For immunostaining, the DCs were incubated with monoclonal antibodies at RT for 1 h, and washed three times for 5 min with PBS. Incubation with PE-labeled secondary antibody was at RT for 30∼45 min, after which the cells were washed as described above. All antibodies were diluted in 3% bovine serum albumin (BSA). The immunostained cells were subsequently mounted in Mowiol (Calbiochem, USA) and examined under fluorescence microscope (Nikon Eclipse E800).

For FACS analysis, DCs were collected as described above, incubated with the purified proteins at RT for 1 h, washed three times for 5 min with PBS, then incubated with anti-HPV16E7 antibody at RT for 30∼45 min, and washed as above. The fluorescence intensity of the purified proteins binding to B7 molecules expressed on DCs was analyzed on FACS Calibur flow cytometer (BD Biosciences, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assays were performed by immobilizing 0.5 μg of HPV16E7 protein in 50 mM $NaHCO_3$, pH 9.6 per microtiter well plate overnight at 4°C. After blocking with 1% PBS, pH 7.5, containing 1% gelatin for 2 h at 37°C, individual protein ligands at a concentration of 5 μg/ml in PBS, pH 7.5, with 0.2% gelatin were added to the wells and incubated for 1 h at RT, and subsequently analyzed for binding using an anti-HPV16E7 monoclonal antibody (Santa Cruz Biotechnology, USA) or sera from immunized mice. In control experiments, the first antigen was omitted from the immobilization step. Incubation with each antibody was carried out in the same solution overnight at 4°C.

Mice

Female C57BL/6 (H-2^b) mice (3∼5 weeks of age) were purchased from Guangzhou Traditional Chinese Medicine University (Guangzhou, China). Mice were housed in filter-top cages under specific pathogen-free conditions and were handled in accordance with the guidelines of Tsinghua University Council on Animal Care.

CTL assay

Cytotoxic T lymphocyte (CTL) assay was performed essentially as described by other workers (Liu *et al*., 2007). Mice were immunized by subcutaneous injection of various immunogens at the nape of the neck. Where indicated, a boost immunization was given one week later. Equimolar of HPV16E7, 16E7-eCTLA4, or an admixture of HPV16E7 and CTLA4 (1:1) were used as immunogens. Control mice received a sham injection of Dulbecco's phosphate-buffered saline (DPBS). Mice were euthanized by CO_2 or cervical dislocation at the desired time points after immunization. Single-cell suspensions of pooled spleens from each group were prepared in CTL medium: RPMI 1640 supplemented with 10% FCS (GIBCO/BRL, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (Sigma-Aldrich, USA), 50 μg/ml gentamicin sulfate (GIBCO/BRL, USA). Splenocytes were restimulated in 10 ml CTL medium by incubating 3×10^7 viable lymphoid cells in the presence of 1 μg/ml E7.49-57 peptide in an upright T25 tissue culture flask. The effector cells were harvested after 7 days and analyzed for cytolytic activity and the release of gamma interferon (IFN-γ) from E7-specific $CD8^+$ T cells. Cytolytic activity was assayed in triplicates in 96-well culture plates by culturing the effector cells with target TC-1 cells (Kenqiang Biotechnique Company, China) expressing HPV16 E7 protein at effector/target (E/T) ratio of 100:1, 33:1, or 11:1. After 4 h of incubation at 37°C/5% CO_2, the 96-well culture plates were centrifuged at 200×g for 5 min using a Beckman G5-6R centrifuge (Beckman Coulter Canada, Canada). One hundred microliters of the culture supernatant was collected from each well into Beckman ready caps, and the released LDH activity was determined by biochemistry analysis using a Beckman Biochemical Analyzer. To determine spontaneous or total release of LDH, target cells were cultured without effector cells in either medium or Triton X-100 (1%, v/v), respectively. Results were expressed as percent specific lysis, which was calculated as $[(LDH_{test}\text{-}LDH_{spont})/(LDH_{total}\text{-}LDH_{spont})]\times100$ (Shan *et al.*, 2006). The control target LDH values were consistently <10% and were subtracted from the results shown. The IFN-γ released in the culture supernatant were determined using the OptEIA mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (eBioscience, USA).

***Ex vivo* analysis of antigen-specific T cells by ELISPOT**

Activated antigen-specific cytotoxic T-cells in splenocytes harvested from immunized C57BL/6 mice were detected using the ELISPOT kit following the instruction manual (eBioscience, USA). Briefly, on day 8 postimmunization, a 96-well nitrocellulose plate was coated with capture antibody, a purified anti-mouse IFN-γ antibody, by incubation overnight at 4°C, and then blocked with complete media. Splenocytes were added to wells at an initial concentration of 10^6 cells/well in a volume of 100 μl and a row of serial dilutions prepared. Cells in a dilution series were either unstimulated or stimulated with E7.49-57 or R187 peptide (1 μg/ml). PMA (5 ng/ml, Sigma-Aldrich, USA) served as positive controls, and R187 peptide and media alone served as negative controls. The plate was incubated overnight at 37°C/5% CO_2. Next day, the plate was incubated with detection antibody (a biotinylated anti-mouse IFN-γ antibody) for 2 h at room temperature. Unbound detection antibody was removed by washing and the enzyme conjugate (Streptavidin-HRP) was added. Following 1 h incubation at room temperature, unbound enzyme conjugate was removed by washing and the plate was stained with an AEC substrate solution for 20 min. The plate was washed, allowed to air dry overnight, and foci of staining were counted using a magnifying lens.

***In vivo* tumor protection**

Mice (10/group) were immunized with PBS, 10 μg HPVE7, 16E7-eCTLA4 and the admixture of HPV16E7 and CTLA4

(1:1) twice with an 8-week interval. The immunizations were staggered so that tumor challenge occurred on the same day for all groups, either 5, 9, or 13 weeks after the last immunization. The mice were challenged subcutaneously with 5×10^5 TC-1 tumor cells/mouse in the hind flank. Tumor incidence and volume were monitored weekly and recorded beginning 9 days after tumor challenge, and monitoring continued for up to 45 days. If a tumor was detected, the two longest dimensions were measured using an electronic digital caliper (Ultra-Cal Mark III Electronic Caliper, Switzerland). Tumor volumes were calculated by width$^2\times$ length$\times$0.5 (Chu *et al.*, 2000). The tumor-bearing mice were euthanized when the tumor volume reached approximately 2,500 mm^3. Differences in tumor incidence between treatment groups 45 days following tumor challenge were evaluated by Fisher's exact test. When the mice were sacrificed, p40 subunits of IL-12 in serum were measured using standard ELISA (eBioscience, USA).

Tumor therapy

Mice in therapy experiments received a tumor s.c. injection with 5×10^5 cells/ml of TC-1 cells in 100 μl of HBSS (Sigma-Aldrich, USA) 10 days before immunization. By day 10, all mice had palpable tumors defined as the presence of a small mass bearing an approximate volume of 0.125 mm^3; and then were treated by PBS, 10 μg HPVE7, 16E7-eCTLA4 or the admixture of HPV16E7 and CTLA4 at 1, 5, or 10 days afterwards. TC-1 tumor growth was monitored and observed their survival rates observed.

Statistical analysis

Nonparametric statistical tests were used (StatXact 4 software, Cytel Corporation, USA). Survival curves were plotted. Data were considered significantly different at $P<0.05$.

Results

Construction and characterization of recombinant HPV16E7-eCTLA4

Before being used as vaccine, specific sites of HPV16 E7 oncogene were mutated (Fig. 1) to both eliminate its host-transforming property and enhance its ability of mediating immune response. To make use of its ability of targeting the B7 molecules on APCs, the extracellular fragment of CTLA4 was amplified from the full length human CTLA4 template. Then linked the two gene fragment via overlap PCR to generate fusion gene HPV16mE7-eCTLA4. HPV16mE7, human CTLA4, and HPV16mE7-eCTLA4 gene were cloned into plasmids and the constructs were verified by DNA sequencing. For *in vitro* and *in vivo* studies, recombinant proteins were expressed from these constructs in *E. coli* strain and purified to homogeneity (Fig. 2A). A lipopolysaccharide elimination procedure was introduced in the purification protocol. The presence of the E7 protein in HPV16mE7-CTLA4, and HPV16mE7 was confirmed by Western blotting using a specific monoclonal antibody (Fig. 2B) or anti-is polyclonal antibody (Fig. 2C). ELISA analysis showed that purified proteins could bind to anti-polyHis antibody (data not shown).

(A) MHGDTPTLHEYMLDLQPETTGLYGYGQLND S//

(B) SEEEDEIDGPAGQAE PDRAHYNIVTFCCK//

(C) GDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPIGSQP

Fig. 1. The amino acid sequence of modified HPV16E7. In the (A) region amino acids 21, 24, and 26 were modified from DLYCYEQ to GLYGYGQ to reduce transformation activity. In the (C) region two zinc finger binding motifs were disrupted by modifications of C61 to G61 and C94 to G94. H-2Db and HLA-A*0201 epitopes were underlined.

The binding of HPV16mE7-eCTLA4 to dendritic cells

To investigate the functional activity of HPV16mE7-eCTLA4, its binding to dendritic cells from mouse bone marrow culture, which express high levels of B7, was tested first. As shown in Fig. 3A, the HPV16mE7-eCTLA4 fusion protein clearly demonstrated its ability to bind to DC cells as detected by anti-HPV16E7 monoclonal antibody; whereas under the same condition, HPV16mE7 did not show binding to DC cells by FACS analysis. Furthermore, immunofluorescence staining with anti-HPV16E7 monoclonal antibody also showed that HPV16E7-eCTLA4 could bind to DC cells,

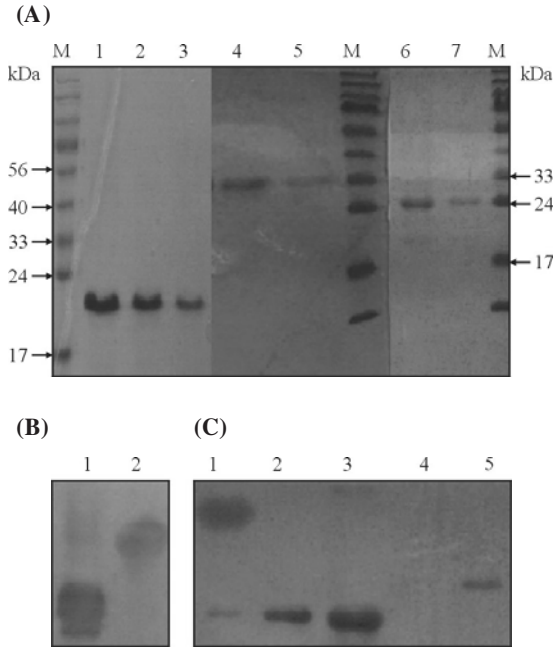

Fig. 2. Protein analysis. (A) SDS-PAGE and staining of purified HPV16mE7, HPV16mE7-eCTLA4, and human CTLA4 proteins expressed in *E. coli.* Lanes 1~3, HPV16mE7; lanes 4 and 5, HPV16mE7-eCTLA4; lanes 6 and 7, CTLA4. Lanes 1~7: proteins eluted with different concentrations of imidazole elution buffer. Lanes 3, 5, and 7, 200 mmol/L imidazole; lanes 2, 4, and 6, 150 mmol/L imidazole; lane 1, 100 mmol/L imidazole. Lanes M, protein size markers. (B) Western blot analysis of HPV16mE7 and HPV16mE7-eCTLA4 using anti-HPV16E7 monoclonal antibody. Lane 1, HPV16mE7; lane 2, HPV16mE7-eCTLA4. (C) Western blot analysis of HPV16mE7, HPV16mE7-eCTLA4, and human CTLA4 using anti-polyHis antibody. Lane 1, HPV16mE7-eCTLA4; lane 2, HPV16E7; lane 3, HPV16mE7; lane 4, blank control; lane 5, CTLA4.

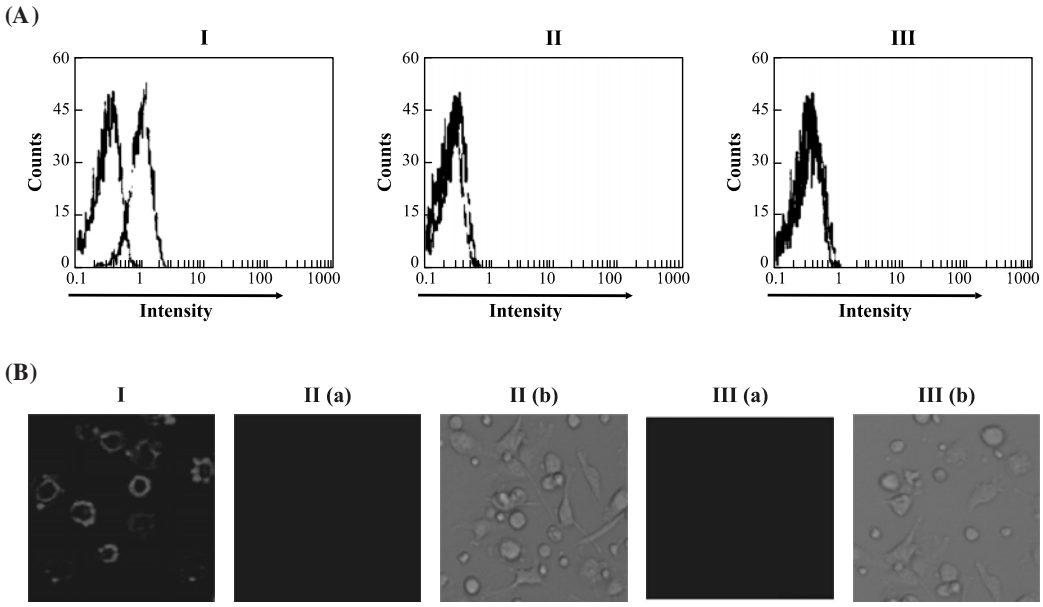

Fig. 3. Protein binding to B7-expressing DC cells. (A) FACS analysis of protein binding to B7-expressing DC cells. After incubation with the following proteins: I, HPV16mE7-eCTLA4; II, human CTLA4; III, HPV16mE7. DCs were analyzed by FACS using anti-HPV16E7 monoclonal antibody to quantify fluorescence intensity as a measure of the binding activity. (B) Immunofluorescence analysis of protein binding to B7-expressing DC cells. After fixing and incubation with the following proteins: I, HPV16mE7-eCTLA4; II, human CTLA4; III, HPV16mE7, DCs were immunostained using anti-HPV16E7 monoclonal antibody and anti-mouse IgG labled with phycoerythrin, and examined under fluorescence microscope, whereas the pictures of II(b) and III(b) were examined under light microscope.

while HPV16mE7 or the mixture of HPV16mE7 and CTLA4 could not (Fig. 3B). Thus, the HPV16mE7-eCTLA4 fusion protein retains the binding activity of CTLA4 for receptor on APCs. Due to the use of anti-HPV16E7 monoclonal antibody, binding of CTLA4 alone to DCs was expectedly not detectable by either FACS or immunofluorescence.

Immune responses induced by HPV16mE7-eCTLA4 fusion protein

To determine the minimal amount of HPV16mE7-eCTLA4 necessary for induction of antibody responses, the animals were given 2 injections of various doses (10, 2, 0.5, or 0.1 μg) of HPV16mE7-CTLA4, and the levels of anti-HPV16E7 Ab induced were assayed at 2 weeks after injection (see 'Materials and Methods'). Mice immunized with 10 or 2 μg HPV16mE7 served as controls. As shown in Fig. 4, mice immunized with HPV16mE7 alone at a dose of either 10 or 2 μg produced relatively low titers of anti-E7 Ab. In contrast, as little as 0.1 μg fusion protein was effective in inducing high titers of anti-E7 Ab. In fact, the anti-E7 titer induced by 0.1 μg HPV16mE7-eCTLA4 fusion protein was comparable to that induced by up to 10 μg HPV16mE7, and was only a little lower than that induced by 10 μg HPV16mE7-eCTLA4 protein (Fig. 4). These results clearly demonstrated that the vaccine efficacy in terms of dosage used in immunization can be dramatically increased by fusing HPV16mE7 to the extracellular moiety of CTLA4.

Tumor protection associated with HPV16mE7-eCTLA4 immunization

To test whether HPV16mE7-eCTLA4 can induce CTL responses against HPV16 E7, C57BL/6 mice were immunized subcutaneously with 10 μg of the different recombinant proteins. Splenocytes were harvested and stimulated *in vitro* with 1 μg/ml of the E7.49-57 peptide. Their ability to lyse TC-1 cells was determined 5 days later using a LDH release assay. Subcutaneous immunization of C57BL/6 mice with fusion protein induced strong and specific CTL responses to TC-1 cells (Fig. 5A). To estimate the frequencies of HPV16 E7-specific splenocytes in mice immunized with recombinant fusion protein, the number of cells producing IFN-γ in response to *in vitro* stimulation with E7.49-57 peptide was

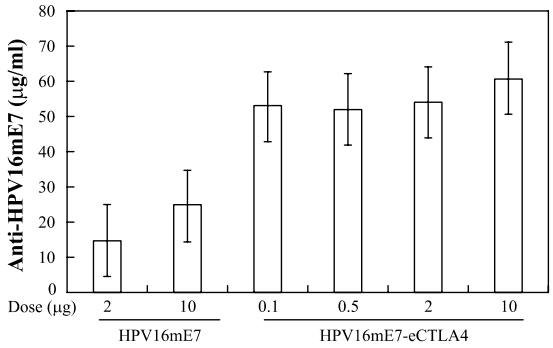

Fig. 4. Effect of HPV16mE7-eCTLA4 dosage on anti-HPV16E7 titer. Mice were immunized twice subcutaneously with various doses of HPV16mE7or HPV16mE7-eCTLA4 and bled 2 weeks after the second immunization. Anti-HPV16E7 titers in immune sera were determined by ELISA. The data are presented as the means and standard deviations for 5 animals of each group.

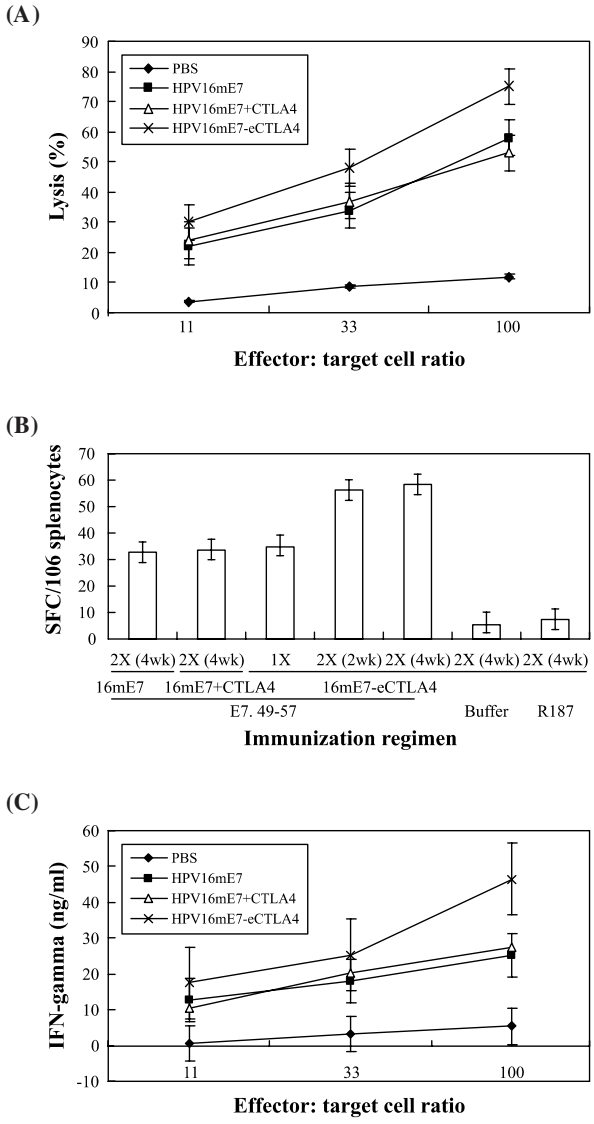

Fig. 5. Responses induced by the vaccine. (A) Assay of CTL response induced by the vaccine. C57BL/6 mice (four per group) were immunized twice with PBS, HPV16mE7, HPV16mE7-eCTLA4, or the admixture of HPV16mE7 and CTLA4 proteins. At 7 days postimmunization, pooled splenocytes from each group were restimulated with peptide E7 49-57 for 7 days and their specific cytolytic activities against TC-1 tumor cells at 11:1, 33:1, and 100:1. Effector/Target ratios were assayed by the released LDH activity. (B) ELISPOT assay of vaccine-induced IFN-γ production. HPV16mE7, the admixture of HPV16mE7 and CTLA4 proteins, or HPV16mE7-eCTLA4 was given as a single immunization (1X) or a prime-boost immunization with a 2- to 4-week interval (2X). The immunizations were staggered so that the IFN-γ-producing cells were assayed *ex vivo* by IFN-γ ELISPOT using freshly isolated splenocytes 7 days after the single or the boost immunization. Data shown are means and standard deviations for two independent experiments. (C) Vaccine-induced IFN-γ secretion. C57BL/6 mice (four per group) were immunized twice with PBS, HPV16mE7, HPV16mE7-eCTLA4, or the admixture of HPV16mE7 and CTLA4 proteins. At 7 days postimmunization, pooled splenocytes from each group were restimulated with E7 49-57 for 7 days and the released IFN-γ in culture supernatant at 11:1, 33:1, and 100:1 E/T ratios were assayed by ELISA.

quantified by enzyme-linked immunospots (ELISPOT). Figure 5B shows that there was only a slight difference in the number of IFN-γ-producing splenocytes obtained from mice immunized with the admixture of HPV16mE7 and CTLA4 as compared with those immunized with HPV16mE7. In contrast, the number of IFN-γ-producing splenocytes obtained was much higher ($P<0.05$) in mice vaccinated with HPV16mE7-eCTLA4. The observed responses were specific as very few splenocytes from these mice produced IFN-γ in the absence of stimulation, i.e. when treated with only media or R187 peptide (Fig. 5B). These results showed that the HPV16mE7-eCTLA4 fusion protein was able to deliver *in vivo* the immunodominant $CD8^+$ T cell into the cytosol of immunocompetent cells and elicit strong CTL responses. Cytokine environment is a major factor in determining the outcome of immunity responses. Therefore the cytokine IFN-γ induced by HPV16mE7-eCTLA4 immunization, with a primary immunization followed by a boost 2 weeks later, was examined. The results showed that the splenocytes from the mice immunized with HPV16mE7-eCTLA4 secreted much more IFN-γ than those from mice immunized with buffer upon restimulation with E7.49-57 peptide (Fig. 5C). HPV16mE7-eCTLA4 is able to stimulate the antigen-specific immune cells to secrete immunestimulatory cytokines, such

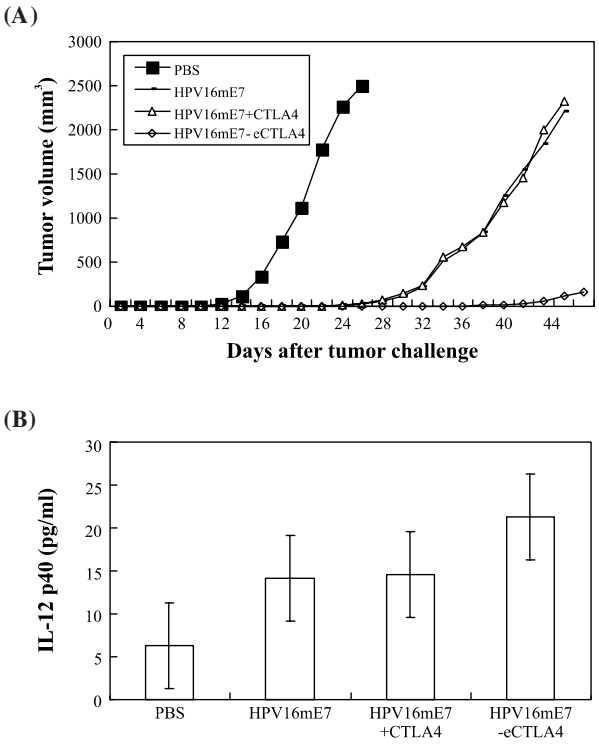

Fig. 6. Tumor volume change and the IL-12 production by immunization. (A) Tumor volume and survival of mice challenged with TC-1 tumor cells after immunization. The mean tumor volume of mice bearing a measurable tumor mass in each group was calculated. The ratio of animals in each group that survived is indicated. (B) The levels of serum IL-12 in mice immunized twice with different proteins. HPV16mE7-eCTLA4 group vs HPV16mE7 or PBS ($P<0.05$).

as IFN-γ, which, in turn, may contribute to a local microenvironment conducive to cell-mediated immunity.

The various proteins containing HPV16E7 were next compared for their efficacy in inducing protection against a lethal tumor challenge. Mice were subcutaneously immunized twice with 10 μg HPV16mE7, HPV16mE7-eCTLA4, or the admixture of HPV16mE7 and CTLA4, and challenged subcutaneously with TC-1 tumor cells expressing HPV16E6E7 protein two weeks following the second immunization. The control mice (injected with PBS alone) did not show any protection – all died within 5 weeks of the tumor challenge, whereas the mice immunized with HPV16mE7, HPV16mE7-eCTLA4 fusion protein, or the admixture of HPV16mE7 and CTLA4 showed protection (Fig. 6A). Our results further showed that the serum IL-12 level was the highest in the HPV16mE7-eCTLA4 group among all the groups (Fig. 6B).

Efficacy of the HPV16mE7-eCTLA4 fusion protein vaccine to treat established tumors

The effects of the HPV16mE7-eCTLA4 vaccine on established tumors were investigated. Groups of 10 C57BL/6 mice received 5×10^5 TC-1 tumor cells each, and after 8 days all mice had palpable tumors defined as the presence of a small mass bearing an approximate volume of 0.125 mm^3. At day 9 the mice were injected with PBS, HPV16mE7, HPV16mE7-eCTLA4, or the admixture of HPV16mE7 and CTLA4 proteins, and the therapeutic treatment was repeated one week days later. Growth of the TC-1 tumors was monitored and rate of survival of the mice observed (Fig. 7). Vaccination with HPV16mE7-eCTLA4 resulted in a decreased tumor growth compared to control groups on day 28 (data not shown), the last time point before mice had to be sacrificed due to overt tumor induced pathology. A repeat of this experiment, combined with the data shown produced a composite P value ($P<0.01$) that indicates that vaccination with the fusion protein vaccine led to a significant decrease in tumor volume compared to vaccination with the controls. Figure 7 indicated that the therapeutic vaccination, although did not eradicate the tumor completely, greatly increased survival of these animals past 75 days.

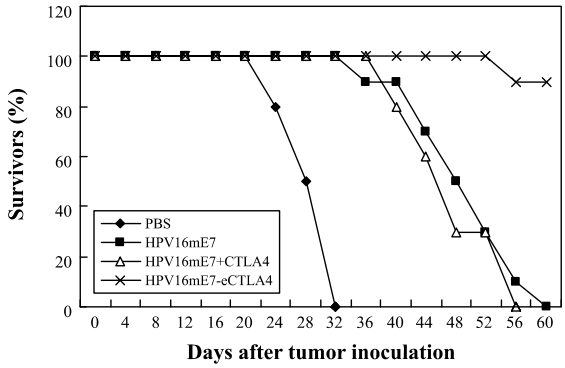

Fig. 7. Effect of vaccination on survival rate of mice with established tumors. Mice with established tumor were immunized twice subcutaneously with 10 μg of HPV16mE7, HPV16mE7-eCTLA4, or the admixture of HPV16mE7 and CTLA4 proteins, or with PBS alone. The percentage of survivors in each group was recorded.

Discussion

Infections with high risk HPV are highly prevalent but generally transient and are believed to be controlled by the cell-mediated immune response since a majority of HPV-induced lesions spontaneously regress (Hopfl *et al.*, 2000; Longworth and Laimins, 2004). The essential factors necessary for HPV-induced malignant progression are largely unknown but may be related to suboptimal or inappropriate T cell responses to HPV antigens during a natural infection. Several therapeutic vaccines that target the E6 and/or E7 proteins have been developed over the recent years (Kanodia *et al.*, 2008). These vaccines aim to control HPV-associated malignancies by activating the patient's own cellular immune response to recognize and kill cancer cells that express the foreign proteins. Several studies have shown that targeting immunogens to various APC populations with Abs against class II MHC, FcgR, and 33D1 or surface Ig can substantially increase immune responses (Snider *et al.*, 1990; Carayanniotis *et al.,* 1991). Immunoconjugated Ag was demonstrated to be more efficiently presented to T cells than Ag alone in an *in vitro* assay. In the present study, fusion protein HPV16mE7-eCTLA4 was generated to serve as therapeutic vaccine for cervical cancer and other HPV16-infection-associated cancers. In our experiments using immunofluoresce microscope and FACS analysis, we found that the HPV16mE7-eCTLA4 fusion protein retained the ability of CTLA4 to bind to B7 molecules expressed on DCs. Low dose HPV16mE7-eCTLA4 fusion protein could induce high titer antibody, IFN- and IL-12 and strong CTL response to protect from tumor challenge. More importantly, this fusion protein at low dose could markedly inhibit established tumor growth and enhance the survival rate of the tumor-bearing mice. These results showed that fusion with CTLA4 was able to convert immunogenic tumor Ag HPV16E7 into a stronger immunogen although anti-HPV16E7 antibody could partly protect from tumor challenges in mice immunized with HPV16mE7 protein or the admixture of HPV16mE7 and human CTLA4, which showed that CTLA4 likely provides such a strong adjuvant activity due to its strong binding affinity to B7 molecules on dendritic cells.

Among the specialized APCs, dendritic cells express high levels of both B7-1 and B7-2 and are particularly important in initiating primary immune responses (Jeffrey *et al*., 2006). CTLA4 binds to both B7-1 and B7-2 with a 20- to 50-fold higher affinity than CD28, another B7-binding receptor on T cells (Linsley and Ledbetter, 1993). Because of the abundance of Langerhans cells and dermal dendritic cells in murine skin, we hypothesize that linking eCTLA4 to HPV16mE7 to improve targeting is very likely to enhance the vaccine immunogenicity via s.c. injection. Indeed, therapeutic immunizations with the fusion protein yielded outstanding results in the tumor rejection experiments when considering the low dosage used for each injection. The biologic activity of CTLA4, that is, the ability to bind B7 molecules, is critical for its enhanced immunogenicity. Together, these results indicated that the strong adjuvant activity of antigen fused with CTLA4 is largely due to its more efficient targeting to

APCs but not the classic carrier effect of the xenogenic Ig or CTLA-4 in the fusion protein (Huang *et al.,* 2000). In our experiments co-immunizing mice with the admixture of HPV16mE7 and CTLA4 resulted in no enhancement of immune responses to HPV16E7, which shows that the physical linkage between HPV16mE7 and CTLA4 is required for the increased immunogenicity of HPV16mE7-eCTLA4. It is therefore probable that the presence of CTLA4 moiety in the fusion protein ensures more efficient targeting of the fusion to APCs, and consequently, leads to more efficient uptake and processing of HPV16mE7-eCTLA4. This is consistent with the previous demonstration that the mutated moiety of B7-molecule-interacting CTLA4 (Y104A) does not bind to B7 molecules. (Huang *et al*., 2000). Other similar strategies, such as linking HSP (Liu *et al*., 2007) and CyaA (Preville *et al.*, 2005), which enhance cell-mediated immunity, to HPV16E7 antigen as therapeutic vaccine, were shown efficacious in mouse models and even in clinical phase II trial.

However, CTLA4-Ig has also been used as an immunosuppressive drug in animal models of transplantation and autoimmune diseases (Jeffrey *et al*., 2006). The immune suppression mediated by CTLA4-Ig is likely through its inhibition of CD28-B7 interactions, which provide an important positive signal for T-cell proliferation and cytokine release. The discrepancy between the immunomodulating effects of CTLA4 in our study and in previous studies is likely due to the number of treatment doses of CTLA4 administered. To achieve maximal immune suppression, a high dose (50∼500 mg) of CTLA4-Ig was applied before Ag treatment and was continued several days thereafter. In our study, HPV16mE7-eCTLA4 was administered only twice with a 2-week interval between immunizations and at much (>5,000 fold) lower doses (10 μg). Using our protocol, it is likely that the B7 molecules on APCs are not significantly blocked. Huang *et al*. (2000) reported that even at a relatively high dose of 50 mg of CTLA4-Ig, B-7 molecules remain available to engage with CD28 and thereby provide a signal for T-cell activation. Our present results and those reported previously by other workers on vaccination with Id-CTLA4, DNA encoding CTLA4-Ig, and DNA vaccines fused with CTLA4 provide lines of evidence that CTLA4-Ig at low levels increases both Ab and T-cell proliferation responses (Boyle *et al*., 1998; Deliyannis *et al.*, 2000; Nayak *et al*., 2003; Tian *et al*., 2004). An alternative explain is, under certain circumstances, CTLA4-Ig can augment immunity. This may occur for two reasons. First, CTLA4-Ig treatment can block ligation of the negative regulator, CTLA4, resulting in enhanced T cell activation (Salomon and Bluestone, 2001). Second, CTLA4-Ig treatment has direct effects on regulatory T cells (Tregs), which are critically important in the control of autoimmunity and of transplant tolerance in many settings (Salomon *et al*., 2000). The development and peripheral survival of Tregs is CD28 dependent; thus, CTLA4-Ig treatment results in a precipitous reduction of Tregs and in some cases exacerbation of autoimmunity (Tang *et al.*, 2003). It is likely to depend on location, timing, and the relevant importance of CTLA4-expressing activated T cells and Tregs in controlling immunity in the different diseases.

Our present results demonstrate that HPV16mE7-eCTLA4 induces potent antigen-specific $CD8^+$ T cells with cytolytic and cytokine-secreting functions, and high titer antibody against HPV16E7, which confer protection against challenge by the antigen-expressing TC-1 tumor cells, inhibit the established tumor growth and improve the survival of the tumor-bearing mice remarkably. In both the protection and therapeutic models, HPV16mE7-eCTLA4 fusion vaccine exhibits greater potency than HPV16E7 alone or an admixture of HPV16mE7 and CTLA4 proteins. Therefore, HPV16mE7-eCTLA4 fusion is a highly promising therapeutic vaccine for the treatment of cervical cancer and other HPV16 infection-caused tumors. The novel approach to augment CTL and Ab responses to the oncoprotien E7 by fusing the extracellular region of CTLA4 to E7 may find expanded applications in developing treatments for a variety of cancers.

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