

Both antigen optimization and lysosomal targeting are required for enhanced anti-tumour protective immunity in a human papillomavirus E7-expressing animal tumour model

Mi Suk Kim¹ and Jeong-Im Sin²

¹Department of Obstetrics and Gynaecology, and ²Department of Microbiology, School of Medicine, Catholic University of Daegu, Namgu, Daegu, Korea

Summary

DNA immunization is a new approach for cancer immune therapy. In this study, we constructed human papillomavirus (HPV) 16 E7 expression vector cassettes and then compared the abilities of these constructs to induce antitumour protection. Lysosome-targeted E7 antigens, and to a lesser degree signal sequence-conjugated and transmembrane region sequence-conjugated E7 antigens in a DNA form, displayed tumour protection significantly higher than wild-type E7 antigens. This enhanced tumour protection was mediated by CD8⁺ cytotoxic T lymphocytes (CTL), as determined by *in vivo* T-cell depletion and *in vitro* interferon- γ (IFN- γ) production. Subsequent co-injection with interleukin-12-expressing cDNA showed insignificantly enhanced antitumour protection. However, E7 codon optimization plus lysosomal targeting resulted in a dramatic enhancement in antitumour protection both prophylactically and therapeutically through augmentation of the E7-specific CTL population, compared to either one of them alone. However, wild-type or codon-optimized E7 antigens without intracellular targeting displayed no protection against tumour challenge. Thus, these data suggest that antigen codon optimization plus lysosomal targeting strategy could be important in crafting more efficacious E7 DNA vaccines for tumour protection.

Keywords: codon optimization; DNA vaccine; human papillomavirus; lysosomal targeting; tumour immunity; cervical cancer

doi:10.1111/j.1365-2567.2005.02219.x

Received 24 February 2005; revised 24 May 2005; accepted 3 June 2005.

Correspondence: Dr J.-I. Sin, Department of Microbiology, School of Medicine, Catholic University of Daegu, 3056-6, Daemyung-4-Dong, Namgu, Daegu, 705-718, Korea.

Email: jsin1964@hanmail.net

Senior author: Dr. J.-I. Sin

Introduction

Human papillomavirus (HPV) 16 infection is a major cause of cervical cancer world-wide.¹ The expression of the HPV oncogenic proteins E6 and E7 is required for tumorigenesis and for maintenance of the tumour state.²⁻⁴ Furthermore, E7-specific immune responses are detected in cervical cancer patients,⁵ suggesting that E7 could be a specific target for immunotherapy against HPV-derived cervical cancers. In this regard, E7-specific prophylactic and therapeutic vaccine strategies have been evaluated in animal model systems. These include direct uses of recombinant E7 proteins plus adjuvants,⁶⁻⁸ DNA vaccines encoding E7,⁹ bacterial/viral vectors expressing E7 or E7

epitope¹⁰⁻¹³ and E7-primed dendritic cells,¹⁴⁻¹⁶ as well as E7 cytotoxic T lymphocyte (CTL) epitopes.¹⁷

In DNA vaccination studies, conjugation of HPV 16 E7 genes with two parts (signal sequence components and endosomal/lysosomal components) of lysosome-associated membrane protein-1 (LAMP-1) resulted in enhanced E7-specific protective immunity against TC-1 tumour cells.^{18,19} In the case of HSV-2 gD antigens, their cellular locations (secreted, cytosolic, or transmembrane types) have also been observed to play a critical role in the induction of immune responses as well as in protection from herpes simplex virus type 2 (HSV-2) challenge.^{20,21} In the studies, a cytosolic form of gD antigens delivered in a DNA form failed to show any protective immunity

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; HRP, horseradish peroxidase; HSV, herpes simplex virus; IFN, interferon; IL, interleukin; i.m., intramuscularly; i.p., intraperitoneally; LAMP, lysosome-associated membrane proteins; OD, optical density; RT-PCR, reverse transcription-polymerase chain reaction; s.c., subcutaneously; Sig, signal; TMR, transmembrane region.

against HSV-2 challenge, as compared to secreted and transmembrane gD types. Furthermore, HPV 16 E7 antigen is known to be a cytosolic protein, highlighting the importance of modulating the cellular location of E7 antigens *in vivo*. Recently, ligation of E7 with the bacterial toxin, calreticulin, viral protein 22 and heat-shock protein 70 has been reported to enhance E7 DNA vaccine potency against TC-1 tumour challenge.^{9,22–24} Dr Wu and his groups also reported that antitumour potency is enhanced by co-injecting E7 DNA vaccines with genes coding for antiapoptotic proteins or serine protease inhibitor.^{25,26} In particular, the lysosomal targeting strategy has been further tested in many other antigen systems.^{27–31} More recently, E7 codon optimization has been reported to increase E7 protein expression *in vitro*.³² Furthermore, conjugation of codon-optimized E7 genes with HPV L1 genes has been shown to induce antitumour protective immunity *in vivo*.³³ However, no effects have been reported for E7 codon optimization plus lysosomal targeting strategy on antitumour protection.

In this study, we constructed different E7 DNA vaccine cassettes and then tested for their abilities to induce protective immunity against TC-1 tumour cells. We observed that lysosome-targeted E7 antigens when used as a DNA vaccine induced significantly greater protection from tumour challenge than either signal sequence- or transmembrane region sequence-conjugated E7 antigens. Furthermore, replacement of wild-type E7 genes with codon-optimized E7 genes in the lysosomal targeting vector resulted in dramatic enhancement in antitumour protection through augmentation of CD8⁺ CTL populations. In contrast, wild-type or codon-optimized E7 antigens without intracellular targeting displayed no protection against tumour challenge. Taken together, these data suggest that the use of antigen codon optimization together with lysosomal targeting could be important in crafting more efficacious prophylactic and therapeutic DNA vaccines for tumour protection.

Materials and methods

Construction of various E7 DNA vaccine types

In the following descriptions the introduced restriction endonuclease recognition sequences are underlined.

For construction of an E7 expression vector, the E7 gene was cloned from pET-E7⁷ using two primers: forward primer 5'-CGGGATCCCCAGGAGGTATGCATGGA-3' (*Bam*HI); reverse primer 5'-GAGCTCGAGGAATTCTTATGGTTTCTG-3' (*Xho*I, *Eco*RI). The resulting E7 genes were digested with *Bam*HI and *Xho*I, and then cloned into a pcDNA3 backbone (Invitrogen, Carlsbad, CA).

For construction of a Sig/E7 expression vector, the signal (Sig) sequence of HSV-2 gB genes was cloned from pTV2-gB (a kind gift from Dr K. L. Jang, Pusan National

University, Korea) using two primers: forward primer 5'-TTGGGATCCATGTCCCCGTTTTACGGCTACCG-3' (*Bam*HI); reverse primer 5'-GAAGATCTCTGCAGGGCCGCCGACGCCACCGC-3' (*Pst*I). The E7 gene was cloned using two primers, forward primer 5'-GAAGATCTCTGCAGATGCATGGAGATACACCT-3' (*Pst*I); reverse primer 5'-CTCGAGGAATTCTTATGGTTTCTG-3' (*Xho*I, *Eco*RI). These were digested with *Bam*HI, *Pst*I and *Xho*I. The resulting *Bam*HI–*Pst*I fragment of gB signal sequences and the *Pst*I–*Xho*I fragment of E7 genes were cloned into a pcDNA3 backbone.

For construction of the Sig/E7/TMR expression vector, the E7 gene was cloned using two primers: forward primer 5'-GAAGATCTCTGCAGATGCATGGAGATACACCT-3' (*Pst*I); reverse primer 5'-CGGAATTCTGGTTCTGAGAACAGAT-3' (*Eco*RI). The transmembrane region (TMR) sequence of the HSV-2 gD genes was cloned from pAPL-gD³⁴ using two primers: forward primer 5'-CGGAATTCGGTATTGCGTTTTGGGTA-3' (*Eco*RI); reverse primer 5'-TTTCTAGAGCTAGTAAAACAATGGCTG-3' (*Xba*I). These were digested with *Pst*I, *Eco*RI and *Xba*I. The resulting *Pst*I–*Eco*RI fragments of E7 genes and the *Eco*RI–*Xba*I fragment of the TMR genes along with the *Bam*HI–*Pst*I fragment of gB signal sequences were then cloned into a pcDNA3 backbone.

For construction of the Sig/E7/LAMP-1 expression vector, a lysosomal targeting sequence was cloned from pcDNA3.1-mouse LAMP-1³⁵ using two primers: forward primer 5'-CGGAATTCAACAACATGTTGATCCCCA-3' (*Eco*RI) and reverse primer 5'-TTTCTAGACTAGATGGTCTGATAGCC-3' (*Xba*I). These were digested with *Eco*RI and *Xba*I. The resulting *Eco*RI–*Xba*I LAMP fragments were substituted for the *Eco*RI–*Xba*I TMR fragment of pcDNA3-Sig/E7/TMR. For construction of pcDNA3-Sig/sE7/LAMP, wild-type E7 genes of pcDNA3-Sig/E7/LAMP were replaced with codon-optimized E7 DNA sequences (sE7) of pIn2-eE7,³² which were generated by polymerase chain reaction (PCR) using two primers: forward primer 5'-GAAGATCTCTGCAGATGGGCGACACCCCCACC-3' (*Pst*I) and reverse primer 5'-CGGAATTCGGGCTTCTGGGAGCAGATG-3' (*Eco*RI), and then digested with *Pst*I and *Eco*RI. All plasmids used in the present study were verified by DNA sequencing analysis.

Confirmation of E7 protein expression *in vitro* by Western blot assay

Renal carcinoma cells (Caki cells; 5×10^5) grown in 60-mm dish plates were transfected with plasmid DNAs using Lipofectamine according to the manufacturer's protocol (Invitrogen). Two days post transfection, cells were collected in 50 μ l lysis buffer (10 mM Tris–HCl, 130 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% Triton X-100) containing protease inhibitors. The protein concentration of each sample was measured using

Bradford reagents (Sigma, St Louis, MO). Either 20 or 40 µg of cell lysates was analysed by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The membrane was pre-equilibrated with TBST solution [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Tween-20] containing 5% skim milk and then reacted overnight at 4° with anti-HPV 16 E7-specific polyclonal sera raised in mice.⁷ After three washes with TBST, the membrane was incubated with anti-mouse immunoglobulin G–horseradish peroxidase (IgG–HRP; Sigma) for 1 hr at room temperature. The immunoreactive protein bands were visualized using the enhanced chemiluminescence detection reagents (Amersham).

Immunization of mice

Female 4- to 6-week-old C57BL/6 mice were purchased from Daehan Biolink, Korea. Mice were injected intramuscularly (i.m.) with 50 µg E7 DNA vaccine cassettes in a final volume of 100 µl 0.25% bupivacaine-containing phosphate-buffered saline (PBS) using a 28-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Fifty micrograms of pcDNA3-interleukin-12 (IL-12)^{34,36} was used for co-injection with E7 DNA vaccine. Plasmid DNA was produced in bacteria and purified by endotoxin-free Qiagen kits according to the manufacturer's protocol (Qiagen, Valencia, CA).

Tumour protection assay

Either 1×10^4 to 5×10^4 or 2×10^5 TC-1 cells were injected subcutaneously (s.c.) into the right flank of C57BL/6 mice for prophylactic and therapeutic vaccine studies. TC-1 tumour cells (a kind gift from T.-C. Wu, Johns Hopkins Medical Institutions) were grown in cRPMI-1640 supplemented with 400 µg/ml of G418. The tumour cells were washed twice with PBS and injected into mice. For therapeutic studies, animals were challenged s.c. with TC-1 tumour cells and injected i.m. with E7 DNA vaccine cassettes the next day. DNA was injected on two further occasions at 1-week intervals. Mice were monitored twice per week for tumour growth, which was measured in cm using a caliper, and was recorded as mean diameter [longest surface length (a) and width (b); $(a + b)/2$]. Mice were killed when tumour size reached more than 2 cm in mean diameter.

ELISA

Enzyme linked immunosorbent assay (ELISA) was performed as previously described.^{7,8,16} In particular, recombinant E7 protein (1 µg/ml in PBS) was used as a coating antigen. For the determination of relative levels of

E7-specific IgG subclasses, anti-murine IgG1, IgG2a, IgG2b, or IgG3 conjugated with HRP (Zymed, San Francisco, CA) were substituted for anti-murine IgG–HRP. To determine IgG isotype levels, sera pooled in an equal volume from 10 mice per group were diluted to 1 : 50 and then reacted with E7 proteins.

Interferon-γ (IFN-γ) assay

A 1-ml aliquot containing 6×10^6 splenocytes was added to the wells of 24-well plates. Then, cells were stimulated with 1 µg recombinant E7 proteins^{7,8} or E7 CTL peptides (amino acids 49–57) containing major histocompatibility complex (MHC) class I epitope¹⁷ per ml. The E7 CTL peptide (RAHYNIVTF) was purchased from Pepton, Korea. After 3 days incubation at 37° in 5% CO₂, cell supernatants were secured and then used for detecting levels of IFN-γ using commercial cytokine kits (Bio-source, Intl., Camarillo, CA) by adding the extracellular fluids to the IFN-γ-specific ELISA plates.

In vivo depletion of CD4⁺ and CD8⁺ T cells

Depletion studies were performed as previously described.^{7,8,16} For *in vivo* cell depletion, anti-CD4 (clone GK1.5) and anti-CD8 (clone 2.43) ascites fluids were generated by injecting hybridoma cells (American Type Culture Collection, Manassas, VA) into pristane-primed nude mice intraperitoneally (i.p.). One hundred microlitres of ascites fluids was administered i.p. on days –3, 0 and 3 of tumour challenge. Antibody treatment resulted in more than 98% depletion of specific CD4⁺ and CD8⁺ T-cell subsets of representative animals over a 3-week period. Depleted mice were subsequently challenged with tumour on day 0.

Statistical analysis

Statistical analysis was carried out using the paired Student's *t*-test and Chi-square test. Values of E7 DNA vaccination alone were compared with values of other E7 DNA vaccine cassettes (Sig/E7, Sig/E7/TMR, Sig/E7/LAMP, Sig/sE7/LAMP). The *P*-values < 0.05 were considered significant.

Results

Construction of E7 DNA vaccine cassettes and their expression

Different E7 DNA vaccine cassettes coding for signal-, TMR-, and LAMP-sequence-conjugated E7 antigens were constructed (Fig. 1a). To confirm whether these DNA vaccines can express their corresponding proteins *in vitro*, cells were transfected with plasmid DNA constructs and

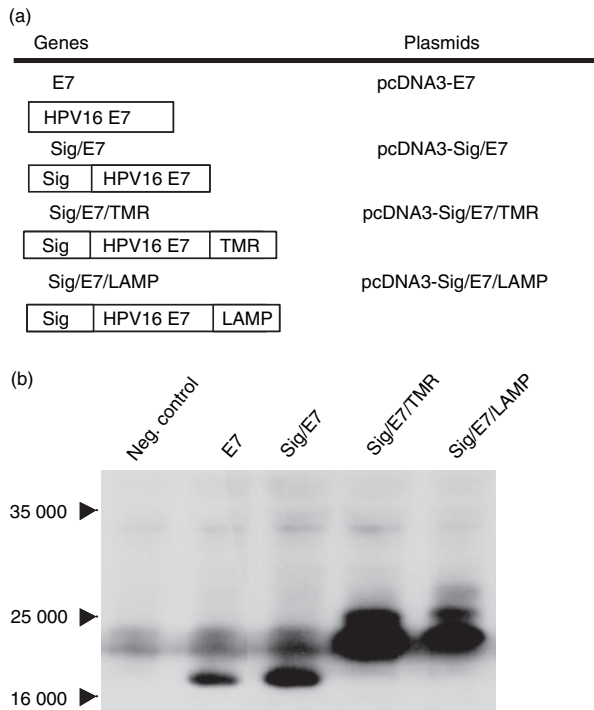


Figure 1. Construction of E7 DNA vaccine cassettes and their expression *in vitro*. (a) HPV 16 E7 genes were conjugated to the signal sequence of HSV-2 gB genes. E7 genes were further conjugated to the transmembrane region (TMR)-targeting sequence of HSV-2 gD genes. Finally, E7 genes were conjugated to the lysosomal targeting sequence (LAMP). (b) Renal carcinoma cells were transfected with E7 DNA cassettes. After 2 days incubation, 40 µg of cell lysates were run on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by Western blot assay. Expression of size-corresponding E7 fusion proteins was confirmed.

then tested for protein expression using Western blot analysis. As shown in Fig. 1(b), each specific E7 protein band was detected in Western blot assay, suggesting that these E7 DNA constructs can express their own proteins *in vitro*.

Anti-tumour effects of E7 DNA vaccine cassettes

To examine which types of E7 DNA vaccines (signal-, TMR-, and LAMP-sequence-conjugated E7 antigens) can induce more potent antitumour protection, animals were immunized with E7 DNA vaccine cassettes at 0, 2 and 6 weeks, followed by challenge with 1×10^4 TC-1 tumour cells per mouse. As shown in Table 1, both signal sequence-conjugated E7 DNA cassettes (pcDNA3-Sig/E7) and lysosomal targeting sequence-conjugated E7 DNA cassettes (pcDNA3-Sig/E7/LAMP) showed 100% protection from TC-1 tumour challenge. However, TMR sequence-conjugated E7 DNA cassettes (pcDNA3-Sig/E7/TMR) showed 70% tumour protection. In contrast, wild-type E7 DNA cassettes (pcDNA3-E7) displayed no

Table 1. Anti-tumour effects of E7 DNA vaccine cassettes

Plasmids	Days post tumour challenge ¹					
	8	15	20	34	42	50
Neg. control (pLacZ)	7	8	8	9	9	–
pcDNA3-E7	6	8	8	9	9	–
pcDNA3-Sig/E7	0	0	0	0	0 ²	0
pcDNA3-Sig/E7/TMR	2	2	3	3	3 ²	3
pcDNA3-Sig/E7/LAMP	0	0	0	0	0 ²	0

Each group of mice ($n = 10$) was immunized i.m. with 50 µg of E7 plasmid DNA cassettes at 0, 2 and 6 weeks and then challenged s.c. with 1×10^4 TC-1 tumour cells per mouse at 8 weeks. Mice were counted for tumour formation over time. This was repeated with similar results.

¹The values represent the number of animals with tumour; the number of animals challenged each time being 10.

²Statistically significant using Chi-square test at $P < 0.05$ compared to negative controls.

protection from tumour challenge in a manner similar to negative controls. Taken together, this suggests that alteration of the intracellular targeting of E7 protein expression can impact on the potency of DNA vaccines against tumour challenge.

Comparison of antitumour functions obtained after tumour cell challenge between pcDNA3-Sig/E7- and pcDNA3-Sig/E7/LAMP-immunized groups

To reaffirm the findings above, we tested two immunization groups (pcDNA3-Sig/E7 and pcDNA3-Sig/E7/LAMP) as these two groups showed 100% tumour protection from 10^4 TC-1 challenge (Table 1). These animals were re-challenged with 2×10^5 tumour cells per mouse (20 time more cells than 1×10^4 cells/mouse). As shown in Fig. 2(a), the pcDNA3-Sig/E7-immunized animals showed similar tumour formation to the negative control groups. However, tumour sizes were far smaller than the control group. In contrast, the pcDNA3-Sig/E7/LAMP-immunized animal group showed no tumour formation 13 days after tumour re-challenge. In particular, a small tumour mass was detectable in some animals from 3 to 13 days following tumour re-challenge, but all of these then regressed. This suggests that lysosomal targeting of an antigen is more useful for inducing antitumour protective immunity against tumour challenge.

CD8⁺ T cells were responsible for antitumour protection

It has been reported that CD8⁺ T cells are responsible for antitumour immunity against TC-1 tumour challenge in E7 DNA vaccination. We next evaluated whether CD8⁺ T cells are responsible for the antitumour protective

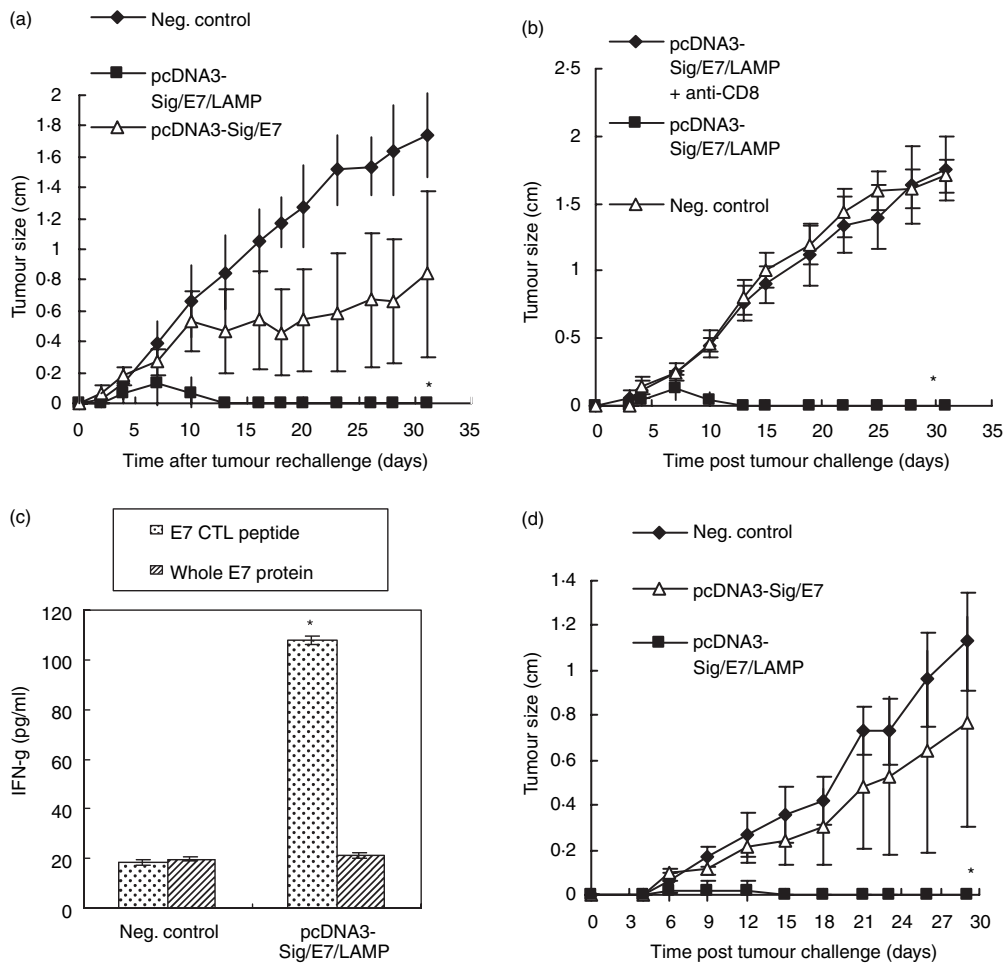


Figure 2. CD8⁺ T-cell (CTL)-mediated antitumour protective immunity by lysosomal targeting of E7 antigens. (a) Two animal groups ($n = 10$, Sig/E7 and Sig/E7/LAMP) showing complete tumour protection for 50 days after tumour challenge (Table 1) were re-challenged s.c. with 20 times more tumour cells (2×10^5 cells/mouse). Tumour size was measured using a caliper twice a week. The mean tumour size [(length + width)/2] in cm was recorded. Values and bars represent mean tumour size and SD, respectively. (b) An animal group ($n = 10$, Sig/E7/LAMP) showing complete tumour protection for 30 days after tumour re-challenge as shown in (a) was divided into two groups and then one group of animals ($n = 5$) was depleted *in vivo* of CD8⁺ T cells. Animals with or without CD8⁺ T-cell depletion were re-challenged s.c. with tumour cells (4×10^5 cells/mouse). Values and bars represent mean tumour size and SD, respectively. (c) pcDNA3-Sig/E7/LAMP-immunized mice showing no tumour in (b) and age-matched control mice were killed for collection of splenocytes. Immune cells were stimulated *in vitro* with $1 \mu\text{g}$ E7 proteins or CTL peptides per ml for 3 days. Cell supernatants were obtained to measure IFN- γ production levels. Values and bars represent mean IFN- γ levels and SD, respectively. (d) Comparison of primary antitumour protection induced by E7 DNA vaccines (Sig/E7 and Sig/E7/LAMP). Each group of animals ($n = 5$) was immunized i.m. with $50 \mu\text{g}$ of pcDNA-Sig/E7 and pcDNA-Sig/E7/LAMP at 0 and 2 weeks. Two weeks after the last DNA immunization, animals were challenged s.c. with TC-1 tumour cells (1×10^4 cells/mouse). Values and bars represent mean tumour size and SD, respectively. This was repeated with similar results. *Statistically significant at $P < 0.05$ using the paired Student's *t*-test compared to pcDNA3-Sig/E7 or control.

abilities obtained after tumour challenge. pcDNA3-Sig/E7/LAMP-immunized animals ($n = 10$) showing 100% protection from tumour re-challenge (see Fig. 2a) were divided into two groups and then animals from one group ($n = 5$) were depleted *in vivo* of CD8⁺ T cells. These mice were re-challenged with TC-1 tumour cells. As shown in Fig. 2(b), animal groups depleted of CD8⁺ T cells showed tumour formation in a manner exactly similar to age-matched negative control animals. However, the animal group without T-cell depletion showed no tumour

formation over time. This confirms that only CD8⁺ T-cell subsets are responsible for antitumour protective immunity. Figure 2(c) shows the induction levels of IFN- γ in five animals showing complete protection against tumour re-challenge. The pcDNA3-Sig/E7/LAMP-immunized animals free from tumour formation (Fig. 2b) were killed and their spleen cells were obtained for stimulation *in vitro* with recombinant E7 proteins or E7 CTL epitopes. As shown in Fig. 2(c), stimulation with E7 proteins resulted in no production of IFN- γ in these

animals in a manner similar to the naive control group. However, stimulation of immune cells with E7 CTL epitopes resulted in induction of IFN- γ production. In contrast, negative control groups induced no production of IFN- γ . This further illustrates a major role of CD8⁺ CTL for antitumour protective immunity.

Comparison of antitumour functions between pcDNA3-Sig/E7 and pcDNA3-Sig/E7/LAMP

We were next interested in comparing the antitumour protective functions of two DNA constructs, pcDNA3-Sig/E7 and pcDNA3-Sig/E7/LAMP. This time, we immunized animals with pcDNA3-Sig/E7 and pcDNA3-Sig/E7/LAMP twice and then challenged them with 1×10^4 TC-1 tumour cells per mouse. As shown in Fig. 2(d), some of pcDNA3-Sig/E7/LAMP-immunized animals displayed tumour formation within 15 days post tumour challenge, but all tumors then regressed. In contrast, animals immunized with pcDNA3-Sig/E7 showed tumour formation in all animals in a manner similar to the negative control group. In particular, the tumour size was smaller in pcDNA3-Sig/E7-immunized animals, as compared with negative controls. This supports the notion that lysosomal targeting of E7 is a more effective approach for inducing E7-specific antitumour protective immunity.

Enhancement of E7 DNA vaccine potency by E7 codon optimization

To increase the E7 DNA vaccine potency, E7 genes of pcDNA3-Sig/E7/LAMP were replaced with codon-optimized E7 genes, and then used for immunization at 0, 2 and 6 weeks, followed by challenges with 5×10^4 TC-1 tumour cells per mouse. As shown in Table 2, pcDNA3-

Table 2. Comparison of antitumour protection between wild-type and codon-optimized E7 genes delivered in the lysosome-targeting vector

Plasmids	Days post tumour challenge ¹							
	8	12	15	19	22	25	30	35
Neg. control (pLacZ)	10	10	10	10	10	10	10	10
Sig/E7/LAMP	7	7	7	6	6	6	6	6
Sig/sE7/LAMP	6	0	0	0	0	0	0	0 ²

Each group of mice ($n = 10$) was immunized i.m. with 50 μ g pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP at 0, 2 and 6 weeks and then challenged s.c. with 5×10^4 TC-1 tumour cells per mouse at 8 weeks. Mice were counted for tumour formation over time.

¹The values represent the number of animals with tumour; the number of animals challenged each time being 10.

²Statistically significant using Chi-square test at $P < 0.05$ compared to pcDNA3-Sig/E7/LAMP.

Sig/E7/LAMP showed 30–40% tumour protection from this tumour challenge dose over time, whereas codon optimization of E7 genes (pcDNA3-Sig/sE7/LAMP) resulted in complete protection from tumour challenge, which is a more than two-fold increase in protection. In contrast, negative controls displayed tumour formation in all animals. We also tested this enhancement of antitumour protection at a higher challenge dose of 2×10^5 TC-1 cells/mouse (Fig. 3a). At this challenge dose, pcDNA3-Sig/E7/LAMP displayed 0% tumour protection. However, pcDNA3-Sig/sE7/LAMP exhibited 100% tumour protection with some tumours formed but then quickly regressed. This is again a dramatic enhancement (0 versus

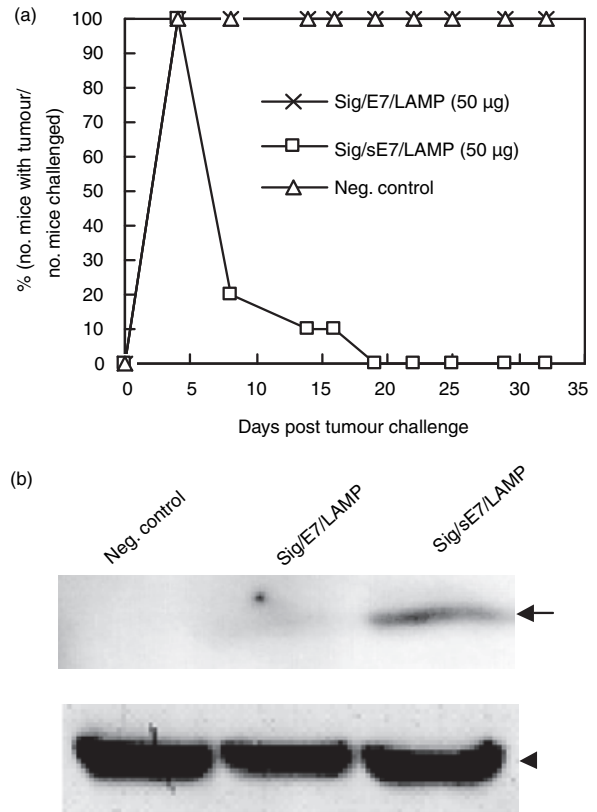


Figure 3. Anti-tumour prophylactic efficacy of pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP at a higher tumour challenge dose (a) and evaluation of E7 protein expression levels *in vitro* (b). (a) Animals were immunized i.m. with 50 μ g E7 DNA vaccines (pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP) at 0 and 2 weeks. At 4 weeks, animals were challenged s.c. with 2×10^5 TC-1 cells/mouse. Animals were checked for tumour formation twice a week. (b) Renal carcinoma cells were transfected with pcDNA3-LacZ, pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP as shown in the Materials and method sections; 20 μ g of cell lysates were run on 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis for Western blot analysis. The filter was re-hybridized with anti-actin antibodies to show that equal amounts of proteins were loaded. Arrow and arrowhead show E7 fusion proteins and actin proteins, respectively. A representative blot is shown.

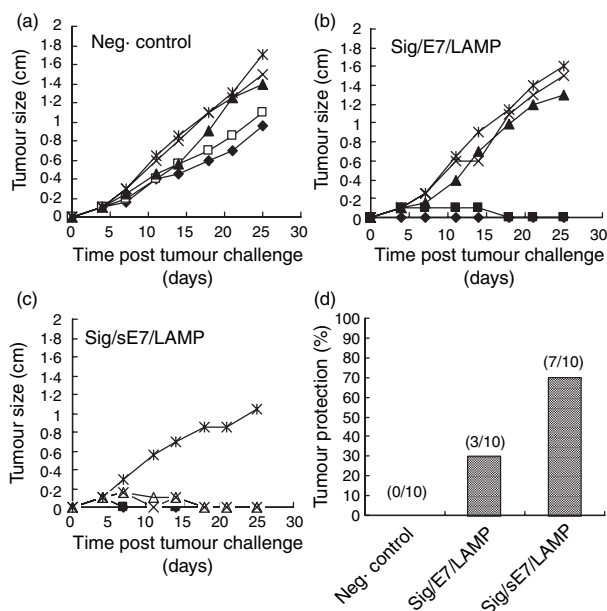


Figure 4. Enhancement of therapeutic efficacy of E7 DNA vaccines by E7 codon optimization. Each group of animals ($n = 5$) was inoculated s.c. with 1×10^4 TC-1 tumour cells per mouse. Next day, animals were immunized i.m. with 50 μg of control DNA vector (a), pcDNA3-Sig/E7/LAMP (b) and pcDNA3-Sig/sE7/LAMP (c), followed by two more injections at 1-week intervals. Tumour size was measured using a caliper twice a week. The mean tumour size [(length + width)/2] in cm was recorded. This was repeated with similar results. (d) Tumour protection rates (%) of mice treated with E7 DNA vaccines. Numbers in (I) represent the number of animals showing complete tumour regression/the total number of animals treated with E7 DNA vaccines.

100%) in antitumour protection by E7 codon optimization plus lysosomal targeting strategy. Figure 3(b) shows expression levels of E7 proteins in Western blot analysis. When cells were transfected *in vitro* with pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP, a significant increase in the E7 protein expression was detected by pcDNA3-Sig/sE7/LAMP, as compared to pcDNA3-Sig/E7/LAMP showing only low expression of E7 proteins, suggesting that increased E7 protein expression levels might be correlated directly to enhanced antitumour protection. We further evaluated therapeutic efficacy of E7 codon optimization. Figure 4 shows the antitumour therapeutic efficacy of codon-optimized E7 genes delivered in the lysosome-targeted form. Animals were inoculated with 1×10^4 TC-1 cells/mouse and then injected i.m. with pcDNA3-Sig/E7/LAMP or pcDNA3-Sig/sE7/LAMP. There was a significant regression of tumour growth in animals injected with pcDNA3-Sig/sE7/LAMP, as compared to those given pcDNA3-Sig/E7/LAMP. Overall regression rate was 0% (none of 10) in vector controls, 30% (three of 10) in pcDNA3-Sig/E7/LAMP and 70% (seven of 10) in pcDNA3-Sig/sE7/LAMP. These data further support the notion that E7 codon optimization plus lysosomal

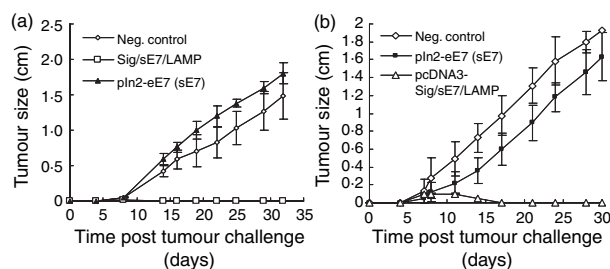


Figure 5. Requirement of lysosomal targeting of codon-optimized E7 genes for enhanced tumour protection. Each group of animals ($n = 5$) was immunized i.m. with 50 μg of control DNA vector, pcDNA3-Sig/sE7/LAMP, and pIn2-eE7 at 0 and 2 weeks. After 4 weeks, animals were challenged s.c. with 2×10^4 (a) and 2×10^5 (b) TC-1 tumour cells per mouse. Tumour size was measured using a caliper twice a week. The values and bars represent mean tumour size and SD, respectively.

targeting exert a critical function in enhancing anti-tumour protective immunity both in prophylaxis and therapeutically.

Lysosomal targeting is required for enhanced antitumour protection

To determine whether both E7 lysosomal targeting and codon optimization are required for enhanced antitumour protection, animals were immunized with pcDNA3-Sig/sE7/LAMP and pIn2-eE7 (a kind gift of Dr Cid-Arregui), and then challenged with 2×10^4 and 2×10^5 TC-1 tumour cells per mouse. As shown in Fig. 5, no tumour was formed in any animal (100% protection) when immunized with pcDNA3-Sig/sE7/LAMP. However, tumours were detected in all animals immunized with codon-optimized E7 genes without intracellular targeting, i.e. pIn2-eE7 (0% protection) over two tumour challenge doses in a manner similar to negative controls. This underscores the importance of both E7 lysosomal targeting and codon optimization for enhanced antitumour protection.

Enhancement of antibody and cellular immune responses by E7 codon optimization

To investigate the levels of E7-specific immune induction by E7 codon optimization, animals were immunized with pcDNA3-Sig/E7/LAMP or pcDNA3-Sig/sE7/LAMP, and then either bled for detection of antibody levels or killed for cellular immune studies. As shown in Fig. 6(a), pcDNA3-Sig/sE7/LAMP induced a detectable amount of E7-specific antibodies, as compared to pcDNA3-Sig/E7/LAMP. In particular, E7 codon optimization resulted in induction of only IgG2b isotypes to a significant level (Fig. 6b). In contrast, other IgG isotypes, such as IgG1, IgG2a and IgG3, were not induced at this

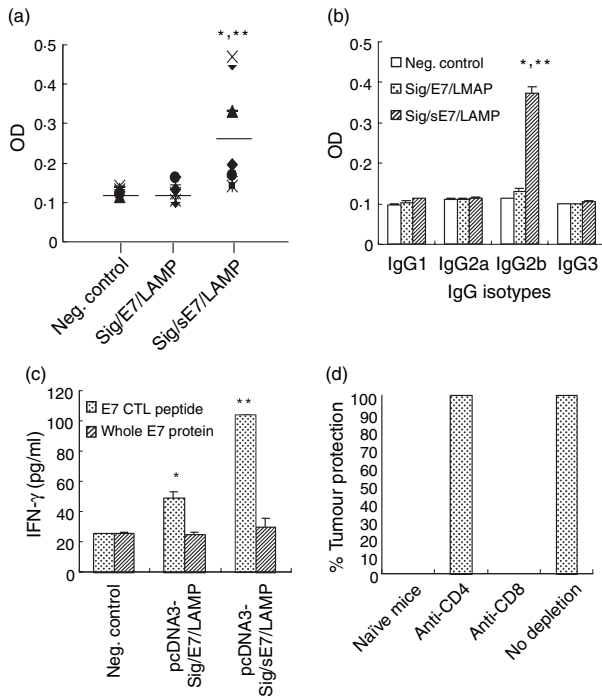


Figure 6. Induction of E7-specific antibody and cellular immune responses by codon optimized E7 DNA vaccines (a, b, c), and effects of T-cell subsets on tumour growth (d). Each group of animals ($n = 10$) was immunized i.m. with 50 μg of pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP at 0, 2 and 6 weeks. Eight weeks after the first DNA injection, animals were bled and sera were diluted to 1 : 50 for ELISA (a). The values and bars represent optical density (OD) values of each serum and the mean, respectively. (b) Equally pooled sera per group were diluted to 1 : 50 for determination of IgG isotype patterns. The values and bars represent means of OD values of equally pooled sera and the SD, respectively. (c) Eight weeks following the first DNA injection, animals were killed and spleen cells were pooled. Splenocytes were stimulated *in vitro* for 3 days with 1 μg HPV 16 E7 proteins or E7 CTL peptides per ml. Cell supernatants were tested for IFN- γ . The values and bars represent means of released IFN- γ concentrations and the SD, respectively. This was repeated with similar results. (d) Animals were immunized i.m. with 50 μg of pcDNA3-Sig/sE7/LAMP at 0, 2 and 6 weeks. After 2 weeks following the third injection, each group of animals ($n = 5$) was depleted *in vivo* of CD4⁺ or CD8⁺ T cells as described in the Materials and methods. Animals were challenged s.c. with 5×10^4 TC-1 cells per mouse and then observed for tumour formation at 20 days following tumour challenge. The values represent the percentage of the number of animals showing no tumour/the total number of animals challenged with TC-1 tumour cells. *Statistically significant at $P < 0.05$ using the paired Student's *t*-test compared to negative control. **Statistically significant at $P < 0.05$ using the paired Student's *t*-test compared to pcDNA3-Sig/E7/LAMP.

injection dose. Figure 6(c) shows IFN- γ production levels of immune cells from animals immunized with pcDNA3-Sig/E7/LAMP and pcDNA3-Sig/sE7/LAMP. In the presence of E7 protein stimulation, IFN- γ production was not detected in immune cells from animals

immunized with either of these two types of DNA vaccines. However, IFN- γ production was detected when immune cells from animals immunized with pcDNA3-Sig/E7/LAMP were stimulated *in vitro* with E7 CTL peptides. In particular, IFN- γ production was enhanced to a more significant level by immunization with pcDNA3-Sig/sE7/LAMP, as compared to pcDNA3-Sig/E7/LAMP. However, little IFN- γ production was detectable in negative controls. These data illustrate that an increase in antigen-specific humoral and cellular (CTL) immune responses can be achieved by replacing wild-type E7 genes with codon-optimized E7 genes in the lysosomal targeting vector.

Tumour protection was mediated by CD8⁺ T cells *in vivo*

We next focused on the possible roles of CD4⁺ or CD8⁺ T cells in protective immunity enhanced by E7 codon optimization against challenge with TC-1 tumour cells. As shown in Fig. 6(d), following vaccination with pcDNA3-Sig/sE7/LAMP, animals were depleted *in vivo* of CD4⁺ or CD8⁺ T cells and then the effects of specific cell populations on tumour protection were tested. When animals previously immunized with pcDNA3-Sig/sE7/LAMP were challenged with tumour cells, complete protection from tumour formation was observed in the absence of immune cell depletion. However, animals depleted of CD8⁺ T cells failed to control tumour formation (0% protection) in a manner similar to negative control animals (0% protection). In contrast, animals depleted of CD4⁺ T cells were protected from tumour growth (in 100% of animals) in a manner similar to the animals without T-cell subset depletion (100% protection). This suggests that CD8⁺ T cells are responsible for enhanced protection against tumour formation. Taken together, these data support the notion that codon-optimized E7 genes in the lysosomal targeting form can enhance protection from tumour growth through effects on CD8⁺ CTL cells *in vivo*.

Discussion

In the present study, we observed that when delivered in a DNA form signal sequence-conjugated, and to a lesser degree TMR sequence-conjugated, E7 antigens induced significantly greater protection from challenge with E7-expressing tumour cells than a native form of E7 antigens. In this case, wild-type E7 antigen failed to show any protection from tumour challenge. Our observation is in line with previous findings in the HSV-2 gD and HCV E2 DNA vaccination model systems.^{20,21,37} In HSV-2 gD studies, deletion of the TMR sequence from gD antigens resulted in enhanced protective immunity against HSV-2 challenge, as opposed to wild-type gD antigens (membrane-targeted form) while deletion of both signal and

TMR sequences generating a cytosolic gD form showed little protective immunity. Similarly, Dr Sung's group³⁷ reported that addition of the TMR sequence to HCV E2 DNA can enhance antigen-specific T-cell responses. These data and ours support the notion that intracellular targeting of antigens can influence the functions of DNA vaccines for immune induction.

It has been reported that conjugation of E7 genes to the lysosomal targeting part of LAMP-1 genes enhances protective immunity against TC-1 tumour cell challenge.^{18,19,38} Effectiveness of lysosomal targeting for the augmentation of antigen-specific immune responses has also been reported in other antigen types.²⁷⁻³¹ We also observed that LAMP sequence-conjugated E7 antigens enhance antitumour protective immunity significantly more than signal or TMR sequence-conjugated E7 antigens, suggesting that lysosomal targeting of an antigen is one of the most effective strategies for induction of antitumour protective immunity. This difference in antitumour protective abilities between DNA constructs is probably the result of different levels of antitumour immune responses induced by each DNA construct. In the case of antitumour protection, CD8⁺ T cells appear to be only an effector T-cell population. This is supported by our observation that when tumour-controllable animals were depleted *in vivo* of CD8⁺ T cells, tumour growth was observed in a manner similar to negative control animals. This is compatible with previous findings that CD8⁺ effector T cells play a major role in protecting animals from challenge with TC-1 tumour cells.^{9-12,39} This is also in parallel with our observation that only upon stimulation *in vitro* with E7 CTL epitopes was IFN- γ production detected from immune cells of tumour-controllable animals previously immunized with lysosome-targeted E7 antigens. Moreover, *in vitro* CD4⁺ and CD8⁺ T-cell subset depletion assay proved that CD8⁺ T cells alone are responsible for E7 CTL epitope-mediated IFN- γ production (data not shown). However, little production of IFN- γ was detectable when immune cells of these animals were stimulated *in vitro* with E7 proteins, suggesting that antigen-specific T helper type 1 (Th1) type CD4⁺ T cells are not induced *in vivo* by E7 DNA vaccines. This is based upon our previous finding that upon stimulation *in vitro* with E7 proteins IFN- γ production is derived from CD4⁺ T cells.⁷ These data confirm that CD8⁺ T cells (CTL) but not CD4⁺ T cells are responsible for antitumour protective immunity induced by lysosomal targeting of E7 genes.

We previously reported that IL-12 as a vaccine adjuvant has a critical role in inducing antiviral and antitumour effects *in vivo*.^{8,34,36} In the present study, however, we failed to see any significant enhancement of antitumour immunity by co-injecting pcDNA3-Sig/E7/LAMP plus pcDNA3-IL-12 (data not shown). This unexpected result might be the result of a lack in E7 protein expression by E7 DNA vaccines *in vivo*. It has been reported that E7

protein expression is hardly detectable by immunoprecipitation methods and Western blot assay, while E7 mRNA expression is detectable to a significant level.⁴⁰⁻⁴² It is also known that E7 protein expression is low and sensitive to degradation in cells.⁴³

We next replaced the E7 gene of pcDNA3-Sig/E7/LAMP with codon-optimized synthetic E7 genes (sE7), and then tested whether E7 codon optimization might impact on antitumour protection against tumour challenge. In our observation, 100% tumour protection was achieved by E7 codon optimization at a higher tumour challenge dose. In contrast, wild-type E7 genes showed 40% protection. This is a more than two-fold increase in tumour protection by E7 codon optimization plus lysosomal targeting strategy. When animals were challenged with more TC-1 cells, E7 codon optimization displayed 100% tumour protection, compared with the wild-type E7 genes showing 0% tumour protection, which is again a dramatic increase in tumour protection. Furthermore, E7 codon optimization resulted in regression of tumours from animals significantly more than wild-type E7 genes in the lysosomal targeting form. Overall regression rate was 0% in vector controls, 30% in pcDNA3-Sig/E7/LAMP and 70% in pcDNA3-Sig/sE7/LAMP, respectively. These suggest that E7 codon optimization plus lysosomal targeting can lead to a significant increase in therapeutic efficacy against tumour growth. This increase in tumour protection appears to be directly related to increased E7 protein expression as we observed higher E7 protein expression by E7 codon optimization *in vitro*. This is also supported by the previous report that E7 codon optimization results in an increase in E7 protein expression *in vitro*.^{32,33} In this case, however, a lack of therapeutic efficacy as compared to prophylactic efficacy might be because tumour cells overgrow before antitumour protective immunity is raised by DNA vaccination in therapy studies. Furthermore, E7 codon optimization resulted in induction of a detectable amount of E7-specific antibodies. Without E7 codon optimization, E7-specific antibodies were hardly detected. This further suggests that the level of antigen expression is a critical factor for inducing antigen-specific antibody production in this model. In particular, E7 codon optimization at the tested vaccine dose resulted in induction of IgG2b isotypes alone. In our previous reports and in other literature, DNA vaccines primarily induce the production of IgG1 and IgG2a isotypes. For instance, HSV-2 gD,³⁶ encephalomyocarditis virus protein 1,⁴⁴ and β -gal-expressing DNA vectors⁴⁵ primarily induce IgG1, IgG2a, and to a lesser degree IgG2b, isotypes. This unique property of IgG2b induction needs to be further investigated. We also observed that upon *in vitro* stimulation with E7 proteins there was little induction of IFN- γ production from CD4⁺ T cells in animals immunized with codon-optimized E7 genes, suggesting that E7-specific Th1-type CD4⁺ T cells cannot be

DNA constructs	Antibody	CD4 ⁺ T (Th1)	CTL (CD8)	Prophylactic responses	Therapeutic responses
pcDNA3-LacZ	-	-	-	-	-
pcDNA3-E7	-	-	-	-	-
pcDNA3-Sig/E7	-	-	+/-	+	-
pcDNA3-Sig/E7/TMR	-	-	+/-	+/-	-
pcDNA3-Sig/E7/LAMP	-	-	+	++	+
pcDNA3-Sig/sE7/LAMP	+	-	+++	+++++	++
pIn2-eE7 (sE7)	ND	ND	ND	-	-

More + represents stronger responses; - represents no responses; ND, not determined.

induced by immunization with codon-optimized E7 genes *in vivo*. This is compatible with the lack of IgG2a production we observed. It has been known that IgG2a formation is dependent on IFN- γ as an IgM-to-IgG2a switch factor and is believed to be typical for Th1-type responses.⁴⁶ This is in contrast to previous reports that targeting E7 expression into the lysosomal compartments is responsible for enhancement of presentation of the E7 antigen by MHC class II molecules.⁴⁷ In our unpublished data, however, lysosomal targeting of E7 antigens induced Th2-type CD4⁺ T-cell responses instead of Th1-type CD4⁺ T-cell responses. However, it is unclear how targeting of an antigen to MHC II pathway results in induction of CD8⁺ T cells. This needs to be further investigated.

Furthermore, gene gun delivery of Sig/E7/LAMP DNA constructs induced E7-specific antibody responses.³⁸ However, we failed to see any induction of E7-specific antibodies by injecting animals *i.m.* with wild-type E7 genes in the lysosomal targeting form. This discrepancy might be ascribed to a difference in the DNA delivery routes tested. However, there was a consistency between previous studies and ours. We observed a significant induction of IFN- γ production from CD8⁺ T cells by immunization with wild-type E7 genes in the lysosomal targeting form. Furthermore, CTL responses were enhanced more significantly by codon-optimized E7 genes in the lysosomal targeting vector cassettes, as compared to wild-type E7 genes in the lysosomal targeting form. This suggests that augmentation of E7-specific CTL responses can be achieved by E7 codon optimization. Thus, these data support that CTL is responsible for enhanced antitumour protection driven by E7 codon optimization plus lysosomal targeting. This is further supported by *in vivo* T-cell subset depletion studies. When CD8⁺ T cells were depleted *in vivo*, no protection from tumour challenge was observed. However, when CD4⁺ T cells were depleted *in vivo*, animals displayed tumour protection in all animals. We also observed that codon-optimized E7 antigens without intracellular targeting delivered in a DNA form fail to induce any protection from TC-1 tumour challenge. This highlights an importance of both E7 codon optimization and lysosomal targeting for enhanced antitumour protection.

Table 3. Levels of antigen-specific immune responses and antitumour protection by E7 expression DNA cassettes

In conclusion, we observe that when delivered in a DNA form a lysosome-targeted form of E7 antigens could be a more potent vaccine type for induction of antitumour protective immunity against tumour challenge, as compared to signal or transmembrane sequence-conjugated E7 antigens. Furthermore, E7 codon optimization in the lysosome targeting system could be more useful for enhancing antitumour protective immunity against TC-1 tumour cells both in prophylaxis and therapeutically through augmentation of antigen-specific CD8⁺ T-cell (CTL) responses. Table 3 summarizes levels of immune responses and antitumour protection by E7 expression DNA cassettes tested in this study. Taken together, this study suggests that in DNA vaccination antigen codon optimization plus intracellular targeting strategy provides an additional option for enhancing E7-specific CTL-mediated antitumour prophylactic and therapeutic immunity against HPV-associated cervical cancer.

Acknowledgements

We wish to thank Dr A. Cid-Arregui for providing codon optimized E7 genes, Dr T. C. Wu for providing TC-1 cells and Dr K. L. Jang for providing HSV-2 gB genes. J.-I. Sin would like to thank Ms Eun-Yu Kim, Ms Young-Ja Park and Dr Sa-Hyun Hong for their technical assistance for this study. This work was supported by Korean Cancer Association Lilly Research Grant.

References

- zur Hausen H. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* 1991; **184**:9–13.
- Scheffner M, Munger K, Bryne JC, Howley PM. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991; **88**:5523–7.
- Werness BA, Levine AJ, Howley PM. Association of HPV type 16 and 18, E6 protein with p53. *Science* 1990; **248**:76–9.
- Dyson N, Howley PM, Munger K, Harlow E. The human papillomavirus-16, E7 oncoprotein is able to bind the retinoblastoma gene product. *Science* 1989; **243**:934–7.
- de Gruijl TD, Bontkes HJ, Stukart MJ *et al.* T cell proliferative responses against human papillomavirus type 16, E7 oncoprotein are most prominent in cervical intraepithelial

- neoplasia patients with a persistent viral infection. *J Gen Virol* 1996; **77**:2183–91.
- 6 Fernando GJP, Murray B, Zhou J, Frazer IH. Expression, purification and immunological characterization of the transforming protein E7, from cervical cancer-associated human papillomavirus type 16. *Clin Exp Immunol* 1999; **115**:397–403.
 - 7 Kim TY, Myoung HJ, Kim JH *et al.* Both E7 and CpG-ODN are required for protective immunity against challenge with human papillomavirus 16 (E6/E7)-immortalized tumor cells: involvement of CD4+ and CD8+ T cells in protection. *Cancer Res* 2002; **62**:7234–40.
 - 8 Ahn WS, Bae SM, Kim TY *et al.* A therapy modality using recombinant IL-12 adenovirus plus E7 protein in a human papillomavirus 16, E6/E7-associated cervical cancer animal model. *Hum Gene Ther* 2003; **14**:1389–99.
 - 9 Hung CF, Cheng WF, Hsu KF *et al.* Cancer immunotherapy using a DNA vaccine encoding the translocation domain of a bacterial toxin linked to a tumor antigen. *Cancer Res* 2001; **61**:3698–703.
 - 10 Lamikanra A, Pan ZK, Isaacs SN, Wu TC, Paterson Y. Regression of established human papillomavirus type 16 (HPV-16) immortalized tumors *in vivo* by vaccinia viruses expressing different forms of HPV-16 E7 correlates with enhanced CD8(+) T-cell responses that home to the tumor site. *J Virol* 2001; **75**:9654–64.
 - 11 Cheng WF, Hung CF, Hsu KF *et al.* Cancer immunotherapy using Sindbis virus replicon particles encoding a VP22-antigen fusion. *Hum Gene Ther* 2002; **13**:553–68.
 - 12 Liu DW, Tsao YP, Kung JT *et al.* Recombinant adeno-associated virus expressing human papillomavirus type 16, E7 peptide DNA fused with heat shock protein DNA as a potential vaccine for cervical cancer. *J Virol* 2000; **74**:2888–94.
 - 13 Londono LP, Chatfield S, Tindle RW *et al.* Immunisation of mice using *Salmonella typhimurium* expressing human papillomavirus type 16, E7 epitopes inserted into hepatitis B virus core antigen. *Vaccine* 1996; **14**:545–52.
 - 14 De Bruijn ML, Schuurhuis DH, Vierboom MP *et al.* Immunization with human papillomavirus type 16 (HPV16) oncoprotein-loaded dendritic cells as well as protein in adjuvant induces MHC class I-restricted protection to HPV16-induced tumor cells. *Cancer Res* 1998; **58**:724–31.
 - 15 Murakami M, Gurski KJ, Marincola FM, Ackland J, Steller MA. Induction of specific CD8+ T-lymphocyte responses using a human papillomavirus-16, E6/E7 fusion protein and autologous dendritic cells. *Cancer Res* 1999; **59**:1184–7.
 - 16 Kim TG, Kim CH, Won EH *et al.* CpG-ODN-stimulated dendritic cells act as a potent adjuvant for E7 protein delivery to induce antigen-specific anti-tumor immunity in a HPV 16 (E6/E7)-associated tumor animal model. *Immunology* 2004; **112**:117–25.
 - 17 Feltkamp MC, Smits HL, Vierboom MP *et al.* Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur J Immunol* 1993; **23**:2242–9.
 - 18 Ji H, Wang TL, Chen CH *et al.* Targeting human papillomavirus type 16, E7 to the endosomal/lysosomal compartment enhances the antitumor immunity of DNA vaccines against murine human papillomavirus type 16, E7-expressing tumors. *Hum Gene Ther* 1999; **10**:2727–40.
 - 19 Smahel M, Sima P, Ludvikova V, Vonka V. Modified HPV16 E7 genes as DNA vaccine against E7-containing oncogenic cells. *Virology* 2001; **281**:231–8.
 - 20 Higgins TJ, Herold KM, Arnold RL, McElhiney SP, Shroff KE, Pachuk CJ. Plasmid DNA-expressed secreted and nonsecreted forms of herpes simplex virus glycoprotein D2 induce different types of immune responses. *J Infect Dis* 2000; **182**:1311–20.
 - 21 Strasser JE, Arnold RL, Pachuk C, Higgins TJ, Bernstein DI. Herpes simplex virus DNA vaccine efficacy: effect of glycoprotein D plasmid constructs. *J Infect Dis* 2000; **182**:1311–20.
 - 22 Cheng WF, Hung CF, Chai CY *et al.* Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J Clin Invest* 2001; **108**:669–78.
 - 23 Hung CF, He L, Juang J, Lin TJ, Ling M, Wu TC. Improving DNA vaccine potency by linking Marek's disease virus type 1 VP22 to an antigen. *J Virol* 2002; **76**:2676–82.
 - 24 Hsu KF, Hung CF, Cheng WF *et al.* Enhancement of suicidal DNA vaccine potency by linking *Mycobacterium tuberculosis* heat shock protein 70 to an antigen. *Gene Ther* 2001; **8**:376–83.
 - 25 Kim TW, Hung CF, Ling M *et al.* Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. *J Clin Invest* 2003; **112**:109–17.
 - 26 Kim TW, Hung CF, Boyd DA *et al.* Enhancement of DNA vaccine potency by coadministration of a tumor antigen gene and DNA encoding serine protease inhibitor-6. *Cancer Res* 2004; **64**:400–5.
 - 27 Raviprakash K, Marques E, Ewing D *et al.* Synergistic neutralizing antibody response to a dengue virus type 2 DNA vaccine by incorporation of lysosome-associated membrane protein sequences and use of plasmid expressing GM-CSF. *Virology* 2001; **290**:74–82.
 - 28 Lu Y, Raviprakash K, Leao IC *et al.* Dengue 2 PreM-E/LAMP chimera targeted to the MHC class II compartment elicits long-lasting neutralizing antibodies. *Vaccine* 2003; **21**:2178–89.
 - 29 Marques ETJ, Chikhlikar P, de Arruda LB *et al.* HIV-1 p55Gag encoded in the lysosome-associated membrane protein-1 as a DNA plasmid vaccine chimera is highly expressed, traffics to the major histocompatibility class II compartment, and elicits enhanced immune responses. *J Biol Chem* 2003; **278**:37926–36.
 - 30 Su Z, Vieweg J, Weizer AZ *et al.* Enhanced induction of telomerase-specific CD4(+) T cells using dendritic cells transfected with RNA encoding a chimeric gene product. *Cancer Res* 2002; **62**:5041–8.
 - 31 de Arruda LB, Chikhlikar PR, August JT, Marques ET. DNA vaccine encoding human immunodeficiency virus-1 Gag, targeted to the major histocompatibility complex II compartment by lysosomal-associated membrane protein, elicits enhanced long-term memory response. *Immunol* 2004; **112**:126–33.
 - 32 Cid-Arregui A, Juarez V, zur Hausen H. A synthetic E7 gene of human papillomavirus type 16 that yields enhanced expression of the protein in mammalian cells and is useful for DNA immunization studies. *J Virol* 2003; **77**:4928–37.
 - 33 Cheung YK, Cheng SC, Sin FW, Xie Y. Plasmid encoding papillomavirus type 16 (HPV16) DNA constructed with codon optimization improved the immunogenicity against HPV infection. *Vaccine* 2004; **23**:629–38.
 - 34 Sin JJ, Kim JJ, Arnold RL *et al.* Interleukin-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1 type CD4+ T cell mediated protective immunity against HSV-2 challenge. *J Immunol* 1999; **162**:2912–21.
 - 35 Guarneri FG, Arterburn LM, Penno MB, Cha Y, August JT. The motif Tyr-X-X-hydrophobic residue mediates lysosomal

- membrane targeting of lysosome-associated membrane protein 1. *J Biol Chem* 1993; **268**:1941–6.
- 36 Sin JI, Kim JJ, Boyer JD, Higgins TJ, Ciccarelli RB, Weiner DB. *In vivo* modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. *J Virol* 1999; **73**:501–9.
- 37 Youn JW, Park SH, Cho JH, Sung YC. Optimal induction of T cell responses against hepatitis C virus E2 by antigen engineering in DNA immunization. *J Virol* 2003; **77**:11596–602.
- 38 Chen CH, Ji H, Suh KW, Choti MA, Pardoll DM, Wu TC. Gene gun-mediated DNA vaccination induces antitumor immunity against human papillomavirus type 16, E7-expressing murine tumor metastases in the liver and lungs. *Gene Ther* 1999; **6**:1972–81.
- 39 Lin KY, Guarnieri FG, Staveley-O'Carroll KF *et al.* Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996; **56**:21–6.
- 40 Greenfield I, Nickerson J, Penman S, Stanley M. Human papillomavirus 16, E7 protein is associated with the nuclear matrix. *Proc Natl Acad Sci USA* 1991; **88**:11217–21.
- 41 Smotkin D, Wettstein FO. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc Natl Acad Sci USA* 1986; **83**:4680–4.
- 42 Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. *Hum Pathol* 1992; **23**:117–28.
- 43 Reinstein E, Scheffner M, Oren M, Ciechanover A, Schwartz A. Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. *Oncogene* 2000; **19**:5944–50.
- 44 Sin JI, Sung JH, Suh YS, Lee AH, Chung JH, Sung YC. Protective immunity against heterologous challenge with encephalomyocarditis virus by VP1 DNA vaccination: effect of co-injection with a granulocyte-macrophage colony-stimulating factor gene. *Vaccine* 1997; **15**:1827–33.
- 45 Raz E, Tighe H, Sato Y *et al.* Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc Natl Acad Sci USA* 1996; **93**:5141–5.
- 46 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; **7**:145–73.
- 47 Wu TC, Guarnieri FG, Staveley-O'Carroll KF *et al.* Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. *Proc Natl Acad Sci USA* 1995; **92**:11671–5.