

## Pre-clinical Immunogenicity and Anti-tumour Efficacy of a Deleted Recombinant Human Papillomavirus Type 16 E7 Protein

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**Abstract.** *Background:* Current vaccination strategies against Human papillomavirus (HPV)-induced ano-genital cancers mostly target E7 from HPV16. However, the oncogenic nature of E7 raises potential human safety issues. Although the modifications abrogating the E7 transforming potential have been well characterized, their effect on E7 immunogenicity has been poorly studied. In this study, we evaluated the vaccine potential of an HPV16 E7 protein deleted from the entire pRb-binding motif. *Materials and Methods:* Purified recombinant deleted (E7 $\Delta$ 21-26) and wild-type (His<sub>6</sub>-E7 and E7WT) E7 proteins were studied in pre-clinical mice models. *Results:* In C57BL/6 mice, E7 $\Delta$ 21-26 formulated with the Quil A adjuvant generated systemic E7-specific cytotoxic T-cell and antibody responses similar to those induced following His<sub>6</sub>-E7/Quil A and E7WT/Quil A vaccinations. E7 $\Delta$ 21-26/Quil A injections efficiently protected animals from challenge with the HPV16-expressing tumours, C3 and TC-1. Moreover, therapeutic vaccination with adjuvant-modified E7 suppressed or significantly decreased C3 tumour outgrowth. *Conclusion:* E7 $\Delta$ 21-26 could represent a safe and efficient vaccine candidate against E7-containing tumour cells.

The contribution of oncogenic Human papillomavirus (HPV) to the development of ano-genital intra-epithelial lesions and cancer has been well established (1,2). Within this HPV subgroup, HPV16 and HPV18, are respectively, the first and second most prevalent types. HPV16 accounts for 57% of the HPV-positive cervical lesions and HPV18 for 17% (3).

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To date, numerous anti-HPV vaccination strategies have been investigated, with most of them targeting either HPV16 or both HPV16 and 18. Both prophylactic and therapeutic vaccines are being developed. The efficacy of prophylactic vaccines, designed to prevent infection, mainly relies on the generation of neutralizing antibodies directed against the viral capsid proteins, L1 or L2. Therapeutic vaccines, to control the progress of established intra-epithelial lesions, are designed to generate cell-mediated immunity against the early viral proteins being expressed by these lesions. While all the early HPV proteins contain epitopes recognized by T-cells, the E7 oncoprotein is presently the most studied vaccine target (4-9) for the following reasons: (i) E7, together with E6, is permanently expressed in cervical lesions while the expression of E1, E2 and E5 is usually lost upon viral DNA integration; (ii) cervical lesions contain a higher amount of E7 than E6; (iii) cervical lesions frequently harbour E6, not E7 variants (10). Prophylactic anti-HPV vaccines present some limitations as they have to be multivalent and have to be given before sexual activity. In contrast, therapeutic vaccines might be monovalent and moreover potentially offer the advantage of generating immunity to several HPV types. Indeed, some studies previously reported the cross-reactivity of the cellular immunity directed against a given HPV type (4,11,12). Using several antigen delivery systems, animal studies have largely demonstrated that HPV16 E7-based vaccination efficiently generates both protective and therapeutic anti-tumour immunity, with E7-specific cytotoxic T-cell response being the main effector mechanism (for review, see 13,14). To date, some of these vaccines have been evaluated in patients; however, while they were usually reported as safe and quite immunogenic, their efficacy still has to be proven (for review, see 15,16).

Vaccination based on HPV16 E7 raises potential safety issues for at least three reasons. The major reason is the oncogenic nature of E7 (17). The second is its tolerogenic

activity and in particular its ability to induce E7-specific peripheral tolerance (for review, see 18). Finally, more recent data have suggested that E7 interferes with the interferon (IFN)- $\alpha$  and  $\beta$  responses and promotes the production of immunosuppressive and angiogenic molecules from human antigen-presenting cells (for review, see 18). Therefore a vaccine based on a non-transforming HPV16 E7 form would be a great advantage, especially for DNA and recombinant live vector vaccines. HPV16 E7 is a 98 amino-acids (aa) nuclear protein binding to a large number of regulatory cellular proteins through three domains: two conserved regions (CR1, CR2) carrying homology with Adenovirus E1A and SV40 T at the amino-terminus and a carboxy-terminal domain containing two Cys-X-X-Cys motifs (17). Specific mutations within each E7 domain have been reported to abrogate its transforming potential: such as, deletion from the aa 6 to 10 in CR1; mutations of C24 to G, C24/26 to G, or deletion from the aa 22 to 26 that abrogate binding to the tumour suppressor, pRb, in CR2; or mutations of C58/91 to G disrupting the Cys-X-X-Cys motifs, in the carboxy-terminal domain (19-21). Although the fusion of E7 with a variety of protein domains has been extensively studied in order to enhance its immunogenicity (13,22-34), to our knowledge only two studies compared the immunogenicity of non-transforming HPV16 E7 forms with that of wild-type E7. E7 variants with C58/91G mutations and the one with D21, C24 and E26 changed to G, were shown to be more immunogenic than wild-type E7. Moreover, the C58/91G mutant underwent more rapid proteasome-mediated degradation (35,36).

In this study, we evaluated a recombinant form of HPV16 E7 protein deleted from the entire pRb-binding motif (aa 21 to 26) produced as a vaccine candidate against HPV16-expressing tumours. In C57BL/6 mice, injection of the adjuvanted E7 $\Delta$ 21-26 generated E7-specific cellular and antibody responses whose intensity was similar to that elicited by vaccines based on a wild-type E7 sequence. Furthermore, we provide evidence that prophylactic and therapeutic E7 $\Delta$ 21-26 vaccinations elicit effective anti-tumour responses.

## Materials and Methods

**Mice and cell lines.** C57BL/6 mice, 6 weeks of age, were purchased from Harlan (Horst, The Netherlands). Protocols were approved by the institutional animal ethics committee. C3 cells (37) were cultured in DMEM (Cambrex Verviers, Belgium) supplemented with 10% FCS, 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, 250 ng/ml fungizone at 37°C in humidified air containing 7% CO<sub>2</sub>. TC-1 cells were grown as previously described (38).

**Vector construction and protein expression.** The pET-28a (+)-E7 vector, encoding an amino-terminal six histidine-tagged E7 protein (E7), was previously described (39). The pPIC9K-E7 vector, expressing wild-type E7 protein in *Pichia pastoris*, was obtained as

follows: the wild-type HPV16 E7 coding sequence was isolated from the pMALcRI-E7 vector (a gift of M. Parmentier, ULB, Belgium) after NcoI and SalI restrictions. The fragment was blunted and then inserted into the pPIC9K plasmid (Invitrogen, Merelbeke, Belgium) linearized with SnaBI (pNIV5102 plasmid). The sequence encoding the E7 protein deleted from the amino-acids 21 to 26 was obtained as follows: the sequence encompassing the E7 coding sequence, including the MF $\alpha$  signal sequence, was recovered after pPIC9K-E7 DNA restriction with BamHI and EcoRI; the blunted fragment was ligated into the pBlue script vector (Stratagene, Amsterdam, The Netherlands) cut with the same enzymes. The resulting plasmid was subjected to site-directed mutagenesis in order to delete the nucleotides encoding E7 amino-acids 21 to 26 and to introduce an EcoRI restriction site downstream to the STOP codon of the E7 sequence. Two oligonucleotides were used: i) 5'-GCAACCAGACA CAACTCAATTAAATGACAGCTCAG-3' (the underlined codons correspond to E7 amino-acids 20 and 27, respectively) and ii) 5'-GTTCTCAGAAACCATAATGAATTCATGTTTCAGGACCCCA CAG-3' (the underlined sequence corresponds to the creation of the EcoRI restriction site). The resulting plasmid, thus carries the MF $\alpha$  factor – E7 $\Delta$ 21-26 DNA sequence. This DNA module was isolated after restrictions with BamHI-EcoRI and inserted at the BamHI-EcoRI sites of pPIC9K to give pNIV5114 plasmid.

Plasmids pNIV5102 and pNIV5114, linearized with BglII, were introduced into the *P. pastoris* strain SMD1168 using the spheroplast transformation method (the procedures were performed according to the manufacturer's instructions – Invitrogen). Transformants were selected for histidine dehydrogenase (His<sup>+</sup>) prototrophy. The screening of His<sup>+</sup> transformants for geneticin (G418) resistance was performed by plating clones on agar containing increasing concentrations of G418 (0.5 to 4 mg/ml). G418 - resistant clones were grown at 30°C in BMGY medium to an OD<sub>600 nm</sub> of 2 and 6. Cells were collected by centrifugation and resuspended in BMGY medium to an OD<sub>600 nm</sub> of 1. E7WT and E7 $\Delta$ 21-26 expression were induced by daily addition of methanol (final concentration 0.5%) during 6 days. The supernatants were collected by centrifugation and stored at -20°C until purification. Aliquots were taken at different times post-induction and assayed for expression and secretion.

**Purification and characterization of recombinant HPV16 E7 proteins.** The methods used for expression and purification of the His<sub>6</sub>-E7 protein have been previously described (26). For purification of E7 $\Delta$ 21-26, spent culture medium was diluted four-fold with water and the pH was adjusted to 7.5. The supernatant was immediately loaded onto a Q sepharose XL column (2.6 x 10 cm) equilibrated in 20 mM Tris-HCl pH 7.5. The column was then washed successively with the equilibration buffer and with the same buffer containing 400 mM NaCl. E7 $\Delta$ 21-26 protein was eluted by a linear NaCl gradient (400 to 650 mM, 15 column volumes). The E7 $\Delta$ 21-26-enriched fractions were pooled and concentrated by ultrafiltration (Amicon YM10 membrane; Millipore, Brussels, Belgium). The purification was achieved by a gel filtration chromatography onto Superdex 200 column (1.6 x 60 cm) equilibrated in PBS buffer pH 7.2. Purified E7 $\Delta$ 21-26 protein was concentrated and stored at -20°C. The purification of E7WT was performed following the purification protocol of E7 $\Delta$ 21-26 protein with the exception that the gel filtration chromatography step was achieved in denaturing conditions. Briefly, the E7WT-enriched fractions from the Q sepharose column were submitted to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation to 60% saturation. The precipitate collected by centrifugation was resuspended in 50 mM Tris-HCl pH 7.5 containing

6M guanidinium hydrochloride, 5 mM EDTA and 10 mM DTT. The sample was then applied onto a Superdex 200 column conditioned in the same buffer. Fractions containing purified denatured E7WT were pooled and the E7WT was renatured by dialysis against PBS pH 7.2. Endotoxins were extracted with Triton X-114 (40) and the remaining endotoxin level was determined using a chromogenic Limulus amoebocyte lysate assay (QCL-1000; Bio-Whittaker, Walkersville, MD, USA). Total protein concentration was determined using the Bradford method (Bio-Rad PROTEIN ASSAY, Nazareth Eke, Belgium) and proteins were subjected to 15% SDS-PAGE and either stained with Coomassie blue or subsequently transferred to nitrocellulose or PVDF membrane for Western blotting or for microsequence analyses respectively, according to the manufacturer's instructions (Amersham, Roosendaal, The Netherlands). Briefly, the membrane was immunoblotted with anti-E7 mouse monoclonal antibody (1:3,000) (TVG710Y; Santa-Cruz) and developed using peroxidase-conjugated sheep anti-mouse IgG (1:4,000) (NA931; Amersham) and chemiluminescence enhancer reagent (ECL Kit; Amersham). The protein NH<sub>2</sub>-terminal amino acid sequences were determined at the picomole level by automated Edman degradation using a Beckman LF3400D protein-peptide microsequencer equipped with an on-line model 126 Gold system microgradient HPLC and a model 168 diode array detector (Beckman Instruments, Analis, Namur, Belgium).

**Cytokine analysis.** Spleen cells, recovered from three mice per group, were pooled and cultured in 96-well U-bottomed plates (5x10<sup>5</sup> cells/well) in DMEM medium supplemented with 1% normal mice serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino-acids, 10 mM HEPES and 0.05 mM 2-ME. The cells were stimulated with E7<sub>49-57</sub> (RAHYNIVTF) and E7<sub>41-62</sub> (PAGQAEPDRAHYNIVTFCKCD) peptides (Eurogentec, Liège, Belgium) in triplicate conditions at 37°C in humidified air containing 7% CO<sub>2</sub>. As controls, cells were incubated with only supplemented DMEM medium. IFN-γ was quantified from 72h cells supernatants using a specific ELISA as previously described (41). The limit of detection was 0.5 U/ml.

**In vivo detection of E7-specific cell lysis.** The method used has been previously described (26). Briefly, the assay consists of injecting *i.v.* to naïve and vaccinated mice a mix of unloaded and E7<sub>49-57</sub>-loaded spleen cells labelled with low or high doses of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) respectively, and in enumerating the remaining CFSE<sup>lo</sup> and CFSE<sup>hi</sup> cells the next day using flow cytometry. The ratio of CFSE<sup>hi</sup>/CFSE<sup>lo</sup> cells were used to calculate the percentage of survival of E7-loaded cells {(CFSE<sup>hi</sup>/CFSE<sup>lo</sup> ratio from a vaccinated mouse divided by the CFSE<sup>hi</sup>/CFSE<sup>lo</sup> ratio from the naïve mouse) x100} and subsequently the percentage of E7-specific cell lysis (100 - % specific cells survival).

**Antibody assay.** Serum levels of anti-E7 IgG were determined by ELISA according to standard procedures using microtiter plates coated with 5 µg/ml of His<sub>6</sub>-E7 protein and peroxidase-conjugated goat anti-mouse IgG (Sigma Aldrich, Bornem, Belgium) for detection. IgG titers are expressed as the reciprocal serum dilution that results in an A490 value of 0.6.

**Tumour protection studies.** Mice were injected twice at three weeks intervals *s.c.* at the base of the tail with no antigen, or equimolar amount of E7Δ21-26 (6.9 µg), E7WT (7.3 µg) or His<sub>6</sub>-E7 (10 µg)

mixed with 15 µg of Quil A (Brenntag Biosector, Hellerup, Denmark). When specified, the E7Δ21-26 dose was doubled (13.8 µg). Two weeks post-immunization mice were challenged with 5x10<sup>5</sup> C3 cells or 4x10<sup>4</sup> TC-1 cells, *s.c.* in the flank. The tumour growth was monitored once a week by measuring the two main tumour diameters.

**Tumour regression studies.** Mice were injected *s.c.* in the flank with 5x10<sup>5</sup> C3 cells and subsequently immunized twice *s.c.* at the base of the tail according to different schedules. Tumour growth was measured twice a week as described above.

**Statistical analysis of the data.** Where indicated, the results of tests of significance are reported as *p* and derived from the Student's *t*-test using a SigmaPlot Statistics 8.0, software package (SPSS, Inc.). For graphical representation of data, y-axis error bars indicate the SD of the data for each point on the graph.

## Results

**Characterization of the E7WT and E7Δ21-26 proteins produced from *P. pastoris*.** On SDS polyacrylamide gel E7WT and E7Δ21-26 migrated as two major (19 and 17 kDa for E7WT, 16 and 14 kDa for E7Δ21-26) and one minor (14 and 10 kDa for E7WT and E7Δ21-26, respectively) forms (Figure 1 A, B). In contrast, His<sub>6</sub>-E7 protein produced in *E. coli* migrated as a single band of 25 kDa (Figure 1A) (26). NH<sub>2</sub>-terminal micro-sequencing analysis clearly indicated that the 19 kDa E7WT form started at E7 Met 1 while the other two forms were truncated E7 derivative products; the 17 kDa form began at either E7 Met 12 or Asp 14 and the 14 kDa form began at E7 Gln 27. The NH<sub>2</sub>-terminal sequence of the 16 kDa, 14 kDa and 10 kDa forms of E7Δ21-26 started respectively at E7 Met12 or Asp14, Gln 27 and Ile 54 (Figure 1C). All bands except the 10 kDa form were detected by E7-specific antibodies (Figure 1B). The E7WT and E7Δ21-26 preparations were highly stable, even after one week at 37°C and contained less than nine endotoxin units per mg (data not shown).

**E7Δ21-26, E7WT and His<sub>6</sub>-E7 generate similar E7-specific cellular and antibody responses.** The immunogenicity of the E7Δ21-26 protein was evaluated in mice. We first assessed its ability to generate an E7-specific IFN-γ response. The spleen cells from mice treated twice with Quil A, E7WT/Quil A, E7Δ21-26/Quil A or His<sub>6</sub>-E7/Quil A were sensitised or not *in vitro* with either the E7-derived MHC class I-restricted E7<sub>49-57</sub> epitope or the E7<sub>41-62</sub> peptide encoding at least one MHC class I- and one MHC class II-restricted epitope (E7<sub>49-57</sub> and E7<sub>48-54</sub>, respectively) (37,42). As shown in Figure 2, the cells from all E7-immunized animals secreted higher levels of IFN-γ in response to the E7 peptides than in medium. The highest amounts of IFN-γ were produced in response to E7<sub>41-62</sub>. Only the animals previously exposed to any form of E7 *in vivo* responded to E7-specific stimulation *in vitro*. Notably,

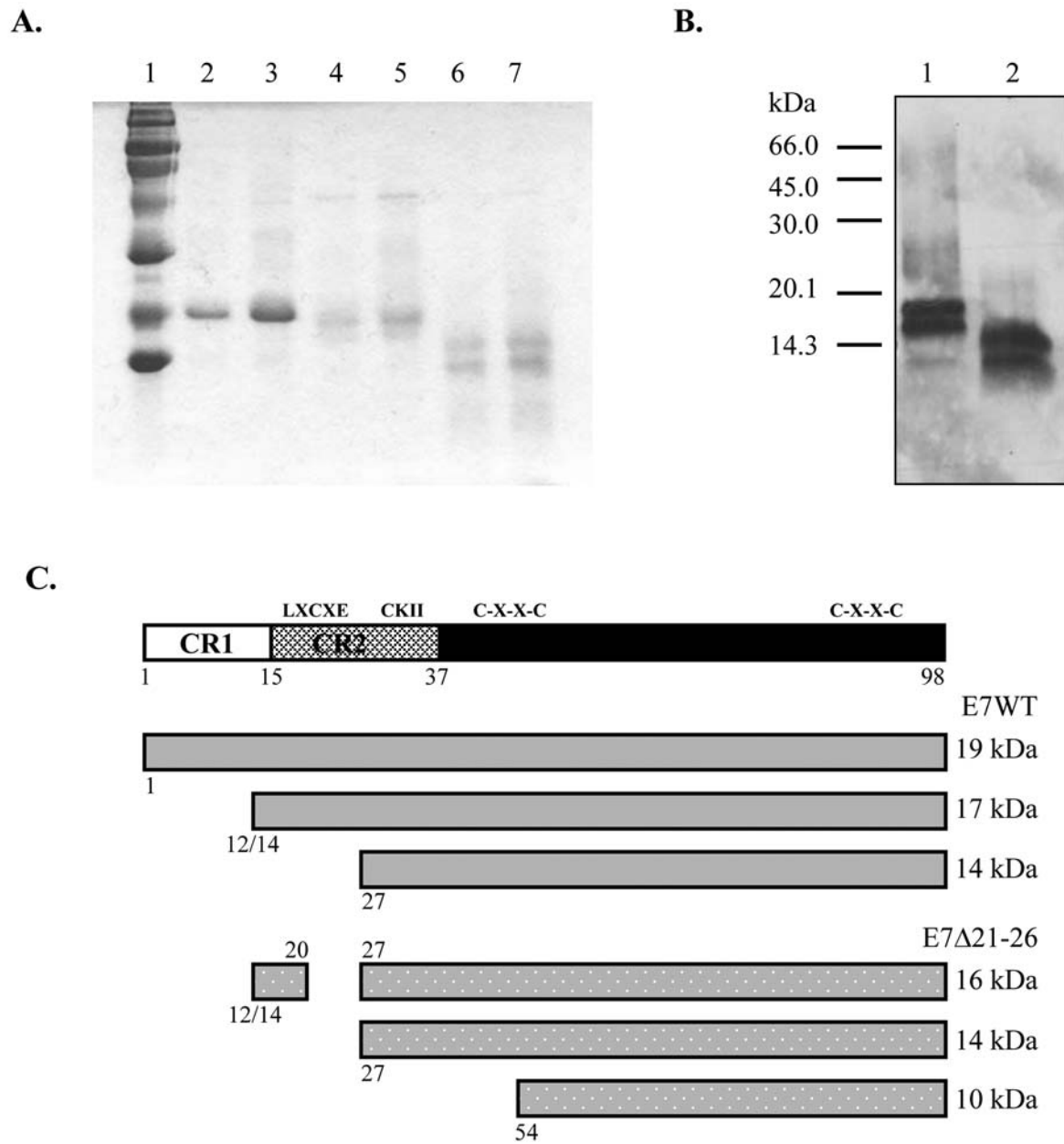


Figure 1. SDS-PAGE, Western blot analysis and structure of E7WT and E7 $\Delta$ 21-26 proteins produced in *P. pastoris*. (A) Coomassie blue-stained SDS-PAGE gel. Mr standards (lane 1), His<sub>6</sub>-E7 (lane 2: 2  $\mu$ g, lane 3: 5  $\mu$ g), E7WT (lane 4: 5  $\mu$ g, lane 5: 10  $\mu$ g) and E7 $\Delta$ 21-26 (lane 6: 5  $\mu$ g, lane 7: 10  $\mu$ g). (B) SDS-PAGE followed by Western blot analysis with anti-HPV16 E7 antibody. E7WT (lane 1: 1  $\mu$ g) and E7 $\Delta$ 21-26 (lane 2: 0.5  $\mu$ g). The position of Mr markers is indicated on the left. (C) Schematic representation of E7WT and E7 $\Delta$ 21-26 products.

animals immunized with E7 $\Delta$ 21-26 displayed an *in vitro* IFN- $\gamma$  response similar to that of the animals vaccinated with proteins based on a wild-type E7 sequence.

Next, we evaluated the ability of E7 $\Delta$ 21-26 to generate E7<sub>49-57</sub>-specific CTL response, in an *in vivo* assay. As shown in Figure 3, E7<sub>49-57</sub>-specific cell lysis was detected in all of the E7-vaccinated animals but, however, with

large individual variations. E7 $\Delta$ 21-26/Quil A-immunized animals generated higher specific lytic activities compared with the E7WT/Quil A-vaccinated mice (Figure 3A). However, over the three independent experiments performed (Table I), only one showed a significant difference (Figure 3A,  $p < 0.05$ ). Moreover, E7 $\Delta$ 21-26/Quil A and His<sub>6</sub>-E7/Quil A vaccinations have

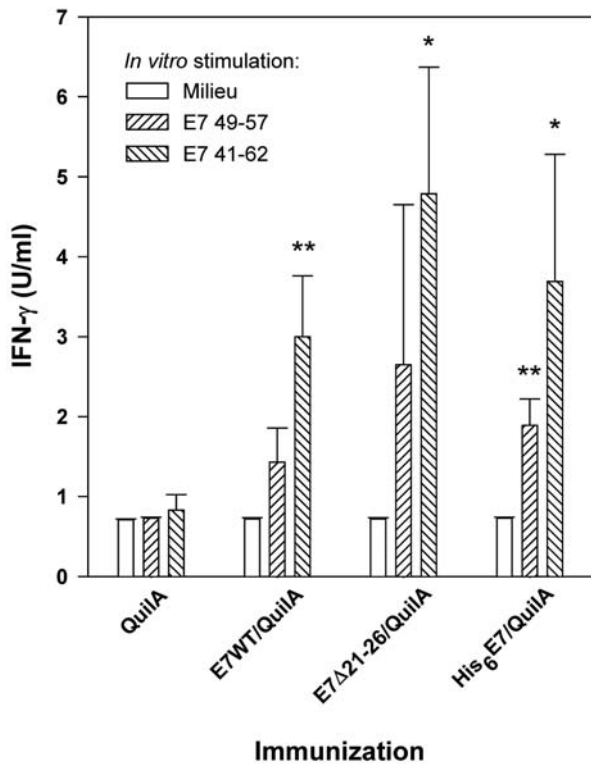


Figure 2. E7-directed IFN- $\gamma$  response following *in vivo* immunization with E7 proteins mixed with Quil A. Groups of three mice were treated twice *s.c.* at two weeks intervals with either Quil A (15  $\mu$ g), E7WT (7.3  $\mu$ g)/Quil A, E7 $\Delta$ 21-26 (6.9  $\mu$ g)/Quil A or His<sub>6</sub>-E7 (10  $\mu$ g)/Quil A. Pooled spleen cells from each group were stimulated or not with E749-57 or E741-62 (9  $\mu$ M). IFN- $\gamma$  content was tested from culture supernatants using ELISA. Results are expressed in U/ml. Cells from E7-immunized mice secreted higher levels of IFN- $\gamma$  following sensitisation with E7 peptides than cells from Quil A-injected mice (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ). Error bars, SDs based on the mean of triplicate values.

induced similar levels of specific cells lysis ( $p > 0.05$ ) (Figure 3B and Table I). Doubling the E7 $\Delta$ 21-26 dose has not significantly increased the E7<sub>49-57</sub>-specific CTL activity (Table I,  $p > 0.05$ ).

Finally, the E7-directed humoral response was evaluated from the sera of immunized and control animals. The pooled data from three independent experiments are depicted in Figure 4. All the His<sub>6</sub>-E7/Quil A- and the majority of the E7WT/Quil A- (4/6) and E7 $\Delta$ 21-26/Quil A-vaccinated mice (8/9) displayed a high anti-E7 antibody response. Similar titers of anti-E7 IgG were detected from the responder animals, whatever the E7 protein preparation used for vaccination ( $p > 0.05$ ). This result further suggested that all these E7 variants induce comparable E7-specific T helper-cell responses. Moreover, like His<sub>6</sub>-E7/Quil A, the E7 $\Delta$ 21-26/Quil A vaccine induced the production of both E7-specific IgG2b and IgG1 (data

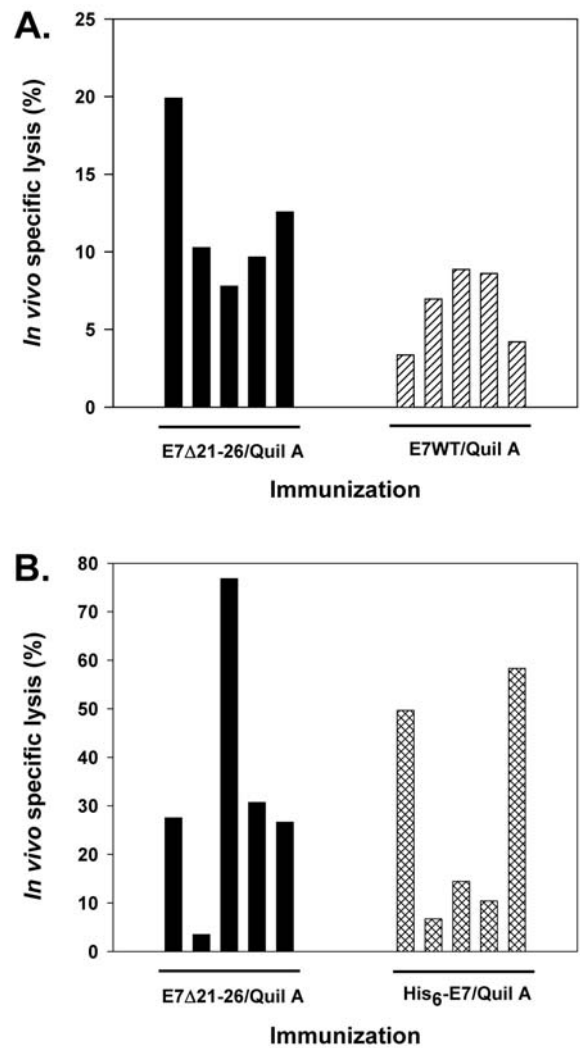


Figure 3. E749-57-directed CTL response following *in vivo* immunization with E7 proteins/Quil A vaccines. (A) One naïve mouse and mice vaccinated twice with either Quil A ( $n = 1$ ), E7 $\Delta$ 21-26 (6.9  $\mu$ g)/Quil A ( $n = 5$ ) or E7WT (7.3  $\mu$ g)/Quil A ( $n = 5$ ) were injected with  $3 \times 10^7$  target cells (see Materials and Methods). Results are displayed as the percentage of E7-loaded cells lysed in each vaccinated mouse, with the mean percentage of lysis from the Quil A-injected mouse subtracted (value was 0.3). E7-directed cell lysis was higher in E7 $\Delta$ 21-26/Quil A- than in E7WT/Quil A-injected mice ( $p = 0.046$ ). (B) Two naïve mice and mice vaccinated twice with either Quil A ( $n = 2$ ), E7 $\Delta$ 21-26 (6.9  $\mu$ g)/Quil A ( $n = 5$ ) or His<sub>6</sub>-E7 (10  $\mu$ g)/Quil A ( $n = 5$ ) were injected with  $4 \times 10^7$  target cells. Results are expressed as in panel A (background value was 1.6). E7-directed cell lysis was not different following E7 $\Delta$ 21-26/Quil A- than His<sub>6</sub>-E7/Quil A injection ( $p = 0.758$ ).

not shown). Taken together, these data demonstrated that E7 $\Delta$ 21-26 was able, as the wild-type E7 forms, to generate strong specific cell-mediated (CTL and Th) and humoral responses *in vivo*.

Table I. E7-specific CTL activity generated *in vivo* following immunization with E7 proteins.

Vaccine antigen (dose)	% <i>in vivo</i> specific lysis			
	E7Δ21-26 (6.9 μg)	E7Δ21-26 (13.8 μg)	E7WT (7.3 μg)	His <sub>6</sub> -E7 (10 μg)
1st assay	12.0 ± 4.7 (n=5) <sup>a</sup>	NT <sup>b</sup>	6.4 ± 2.5 (n=5)	22.4 ± 7.4 (n=5)
2nd assay	33.0 ± 26.8 (n=5)	NT	NT	27.9 ± 24.2 (n=5)
3rd assay	29.7 ± 21.1 (n=4)	47.7 ± 30.3 (n=4)	15.4 ± 7.5 (n=4)	24.8 ± 16.4 (n=3)
4th assay	10.7 ± 5.7 (n=5)	13.8 ± 6.9 (n=5)	7.1 ± 5.6 (n=5)	NT

C57BL/6 mice were immunized *s.c.* at the tail base with E7 proteins mixed with Quil A (15 μg) or Quil A alone, twice at 2-week intervals. Seven days post-vaccination mice were injected with target cells (see Methods). Naïve mice were also injected as a reference. Results are expressed as the mean of the percentages of E7-loaded cells lysed from each vaccinated mouse, with the mean percentage of lysis from the Quil A-injected mice subtracted (values were 0.3, 1.6, 2.8 and 0.3 for experiments 1, 2, 3 and 4, respectively). <sup>a</sup>Number of injected mice; <sup>b</sup>not tested.

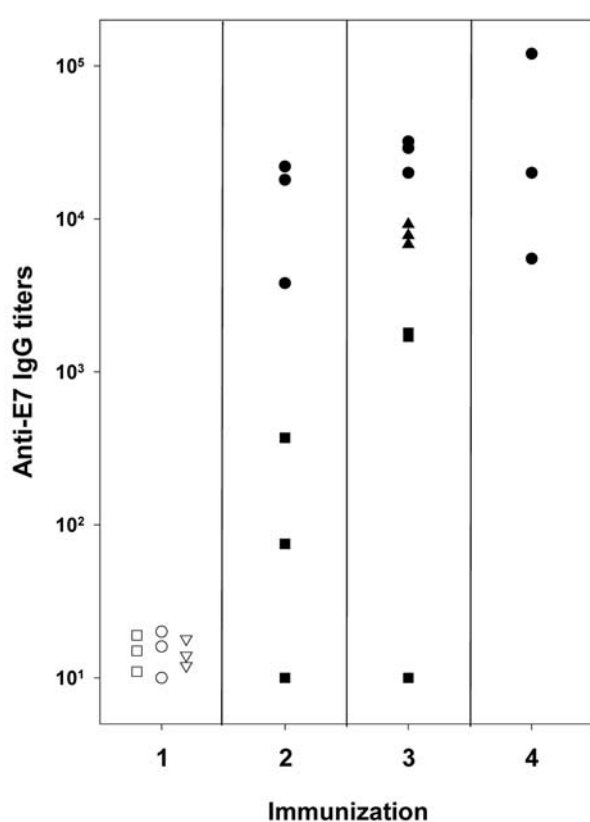


Figure 4. Anti-E7 IgG titers following immunization with E7 proteins formulated with Quil A. Mice were treated twice *s.c.* with Quil A only (1), E7WT (7.3 μg)/Quil A (2), E7Δ21-26 (6.9 μg)/Quil A (3) or His<sub>6</sub>-E7 (10 μg)/Quil A (4). Mice were bled 7 to 10 days post-immunization. Individual mice sera were analysed for anti-E7 IgG reactivity. Anti-E7 IgG titers are expressed as the reciprocal of serum dilution that results in an A490 value of 0.6. Data presented for each treatment result from one to three independent experiments.

The E7Δ21-26/Quil A vaccine efficiently prevents the outgrowth of HPV16 E7-expressing tumours. We next assessed tumour resistance after prophylactic vaccination with

adjuvanted E7Δ21-26 using two well characterized E7-expressing tumour models, C3 and TC-1 (37,38). Both tumour cell lines express similar levels of surface MHC class I molecules but TC-1 cells contain higher amount of E7 protein (at least 6-fold), suggesting that this cell line might be more immunogenic than C3 (data not shown). Compared with C3, TC-1 cells are tumorigenic at a lower dose (at least 5-fold) and possess a higher capacity to metastasize in the lung (43). Tumour resistance was evaluated after two injections of E7Δ21-26/Quil A. Groups of mice were also immunized with either E7WT/Quil A or His<sub>6</sub>-E7/Quil A, as controls. The data from five independent experiments, summarized in Table II, show that the majority (92%) of the E7Δ21-26/Quil A-vaccinated mice remained tumour-free after challenge with C3 cells. Similar levels of protection were obtained with E7WT/Quil A (75%, *p*>0.05) and His<sub>6</sub>-E7/Quil A (100%, *p*>0.05). The E7Δ21-26/Quil A immunization also efficiently prevented the outgrowth of TC-1 tumour (83.3% of tumour-free mice) (Figure 5). Tumours grew in all of the adjuvant alone-injected mice, whatever the type of tumour cells used (Table II and Figure 5). The protective immunity elicited by E7Δ21-26/Quil A was long-lasting as 75% of the mice that resisted to a first challenge with C3 remained tumour-free upon a subsequent tumour challenge performed 3 months later (data not shown). It is unlikely that resistance to the second challenge was elicited in response to the first one. Indeed, mice previously injected with irradiated C3 cells were not resistant to challenge with live C3 cells; these mice developed tumours growing at the same rate as those of the naïve mice challenged as control (data not shown). Taken altogether, these results suggest that E7Δ21-26 is an efficient inducer of tumour resistance *in vivo*.

The E7Δ21-26/Quil A vaccine generates therapeutic anti-tumour effects. The therapeutic anti-tumour potential of the E7Δ21-26/Quil A vaccine was evaluated in animals that had been previously implanted with C3 tumour cells. Tumours

Table II. Tumour protection generated following E7Δ21-26/Quil A immunization.

Immunization	Number of tumour-free mice/ number of immunized mice				
	1st assay	2nd assay	3rd assay	4th assay	5th assay
E7Δ21-26/Quil A	8/8	7/8	7/8	5/6 <sup>a</sup>	6/6 <sup>a</sup>
E7WT/Quil A	NT <sup>b</sup>	6/8	NT	NT	NT
His <sub>6</sub> -E7/Quil A	8/8	NT	NT	6/6	NT
Quil A	0/8	0/8	0/8	0/6	0/6

C57BL/6 mice were immunised twice *s.c.* at the base of the tail with E7Δ21-26 (6.9 μg), E7WT (7.3μg) or His<sub>6</sub>-E7 (10 μg) mixed to Quil A or with Quil A alone. Two weeks post-immunization mice were challenged with C3 cells. <sup>a</sup>Mice received 13.8 μg of E7Δ21-26 instead of 6.9 μg; <sup>b</sup>not tested.

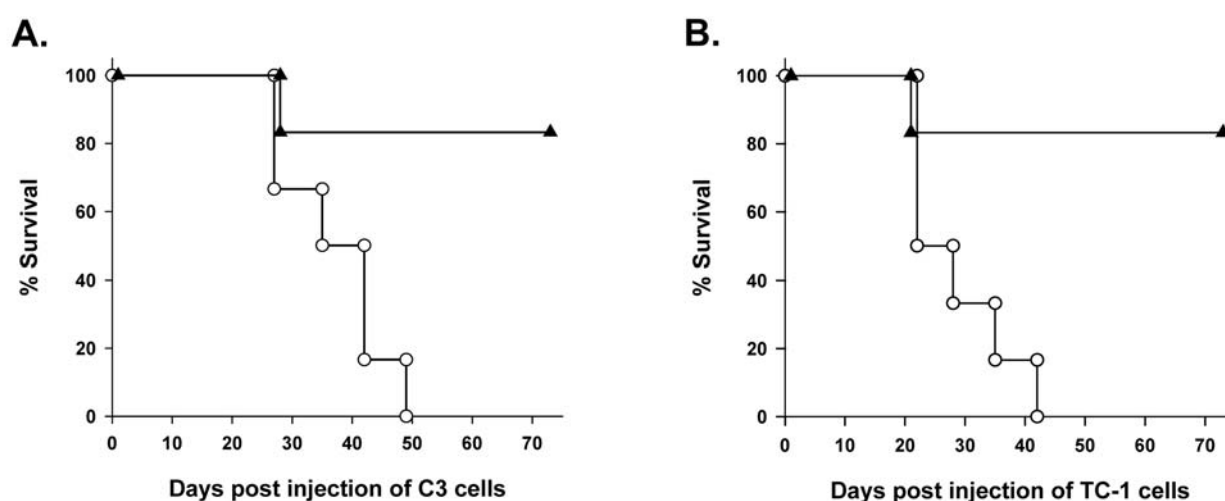


Figure 5. Protective anti-tumour immunity generated following E7Δ21-26/Quil A injections. Groups of 12 mice were injected twice *s.c.* with Quil A (15 μg) (○) or E7Δ21-26 (13.8 μg)/Quil A (▲). Two weeks post-immunization, one half of the mice from each group was challenged with C3 cells (A) and the other half with TC-1 cells (B). Survival, in percent, is expressed relative to time post-challenge.

began to grow at similar rates in all the mice, as shown in Figure 6. However, significant tumour regression was observed in the E7Δ21-26/Quil A-treated mice using two schedules of vaccine injection. When vaccination was performed 3 and 10 days post tumour challenge, 1 out of 8 mice showed complete tumour eradication and 5 a partial response (Figure 6B). When the treatment was delayed for 2 days, 2 out of the 6 animals became tumour free and 2 showed a partial response (Figure 6C). All of the Quil A-treated mice developed large tumours (Figure 6A). Similar results were obtained in three independent experiments (Table III). The compiled data show that the E7Δ21-26/Quil A vaccine had generated anti-tumour responses in 73% of the animals, while no response was measured in the control animals. Of note, the E7Δ21-26/Quil A vaccine significantly extends the survival of the partial and non-regressors animals (Table III).

## Discussion

The set up of a therapeutic vaccine against HPV-induced ano-genital intra-epithelial neoplasia is the focus of much research. Most of the immunotherapeutic strategies under evaluation target E7 from HPV16, the most prevalent oncogenic mucosal genotype. The well-characterized oncogenic nature of E7, as well as its more recently discovered tolerogenic and immunosuppressive activities, raise potential safety issues when considering its administration to humans. Therefore, we find it surprising that the immunogenicity of non-transforming E7 forms still has been poorly documented. Bournnell *et al.* (44) studied the immunogenicity of a recombinant vaccinia virus encoding for both HPV16 and 18 E6/E7 fusions with key mutations in each viral protein (TA-HPV). For HPV16 E7, C24 and E26 were changed to G in order to abrogate its

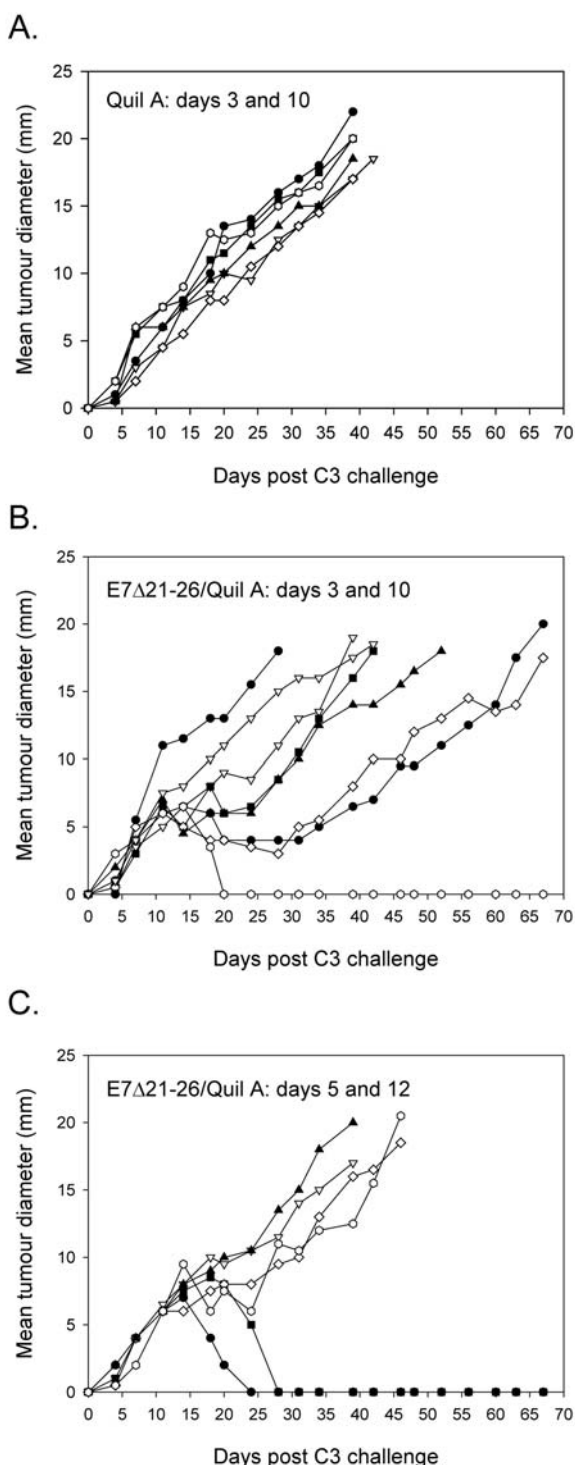


Figure 6. Therapeutic anti-tumour activities generated following E7Δ21-26/Quil A treatments. Mice were challenged s.c. with C3 cells. Three (A, B) or five days later (C), mice were injected s.c. either with Quil A (15 μg) (n=6) (A) or with E7Δ21-26 (13.8 μg)/Quil A (15 μg) (B, n=8) (C, n=6). Mice were boosted one week after the first injection. Each line represents the mean diameter of an individual C3 tumour, measured at the given time-points and displayed in mm.

binding to pRb. The authors showed that TA-HPV elicited similar E7-directed CTL activity than a recombinant vaccinia virus encoding wild-type E7, in C57BL/6 mice. In addition, TA-HPV also generated T-cell responses to HLA-A2-restricted E7 peptides in patients (45). Using DNA vaccination, two pre-clinical studies reported the interesting finding that non-transforming point mutated E7 forms were more immunogenic than the native form (35,36). These data underline the interest in further studying of the immunogenicity of modified E7 sequences.

In this report, we evaluated a safe protein-based prophylactic and therapeutic vaccine against HPV16 in a mouse model. For the production of a non-transforming E7 form as vaccine antigen, we used an E7 sequence deleted from the nucleotides encoding for the entire pRb-binding motif (aa 21 to 26). We showed that this deleted E7 sequence led to the production of various amino-terminal truncated E7 products in *P. pastoris*, not to the expected 92 aa product. According to previous studies, these amino-terminal truncations abrogate by themselves the transforming potential of E7 (19,20). Moreover, a recent study reported that *in vitro* this antigenic preparation was devoid of immunosuppressive activity (46). With the native E7 sequence, *P. pastoris* produced both the full-length oncoprotein and truncated products starting either at Met 12 or Asp14. Braspenning *et al.* (47) previously made similar observations for the expression of E7 from the yeast *Schizosaccharomyces pombe*. While we have not investigated the molecular mechanisms underlying the amino-terminal truncations of E7, some hypothesis might be formulated. Braspenning *et al.* (47) have suggested the use of either Met 1 or Met 12 for expression of wild-type and point mutated E7 from *Sz pombe*. Moreover, HPV-16 E7 was recently shown to be a novel substrate of the ubiquitin pathway that is targeted for degradation *via* NH<sub>2</sub>-terminal ubiquitination. According to a set of rules determined for yeast proteins and based on the three NH<sub>2</sub>-terminal residues, E7 expressed in *Pichia pastoris* should not be acetylated which consequently exposed the E7 NH<sub>2</sub>-terminus and favours NH<sub>2</sub>-terminal ubiquitination (48). It must be pointed out that the deletion of the first 11 aa of E7 has been reported to stabilize the protein (49).

Adjuvanted E7Δ21-26 preparation generates effective anti-tumour immune responses. Non-adjuvanted proteins are rather weak immunogens, typically inducing humoral responses while the effectors of anti-tumour immunity are mainly CTLs (50). We therefore formulated E7Δ21-26 with the Quil A saponin known to be a potent enhancer of effector T-cells directed to subunit antigens (51). We found that E7Δ21-26/Quil A induced long-lived protection against two E7-expressing tumours, C3 and TC-1, in most of the animals. When tested in a therapeutic setting, the E7Δ21-26/Quil A formulation generated significant anti-tumour responses, ranging from growth delay and long-term survival



Table III. Therapeutic anti-tumour effects induced by E7Δ21-26/Quil A.

Assays	Treatment dates	E7Δ21-26/Quil A				Quil A			
		CR	PR	NR	Mean survival time <sup>a</sup>	CR	PR	NR	Mean survival time <sup>a</sup>
1st assay <sup>b</sup>	Days 2 and 7	1/8	7/8	0/8	42 ± 8	0/8	0/8	8/8	26 ± 3
2nd assay <sup>c</sup>	Days 5 and 12	2/8	2/8	4/8	35 ± 8	0/6	0/6	6/6	26 ± 1
3rd assay	Days 5 and 12	2/6	2/6	2/6	42 ± 4	NT <sup>d</sup>	NT	NT	NT
	Days 3 and 10 <sup>c</sup>	1/8	5/8	2/8	48 ± 15	0/6	0/6	6/6	39 ± 1

C57BL/6 mice pre-implanted with C3 cells (day 0) were immunized twice *s.c.* at the tail base with E7Δ21-26 (<sup>b</sup>6.9 μg or <sup>c</sup>13.8 μg) mixed with Quil A (15 μg) or Quil A alone. CR: complete regression; PR: partial regression or stable lesion size for ≥ 10 days with a diameter < 11 mm; NR: no regression. <sup>a</sup>Mean survival time for PR and NR. Mean survival time for E7Δ21-26/Quil A- versus Quil A-treated mice,  $p=0.0001$ ,  $p=0.015$  and  $p>0.05$  for 1st, 2nd and 3rd assays, respectively. <sup>d</sup>not tested.

benefit to complete regression of tumours. We also characterized the E7-directed immune responses elicited following E7Δ21-26/Quil A vaccination. Both significant cellular and humoral responses were detected whose intensity was similar to those resulting from the vaccination with proteins based on a wild-type E7 sequence. In particular, the induction of the expected immune phenotype was proven by the detection of E7-specific CTL, IFN-γ and IgG2b responses. Some of our preliminary findings suggest that E7Δ21-26 might even be a more effective vaccine antigen than His<sub>6</sub>-E7 made of a wild-type E7 sequence. First, E7Δ21-26/Quil A generates long-lasting anti-tumour immunity in higher percentage of mice than His<sub>6</sub>-E7/Quil A (data not shown). Second, E7Δ21-26/Quil A induces better protection against TC-1 (data not shown). Although the immunogenicity of non-transforming E7 forms point mutated within either CR2 or the carboxy-terminal region has already been demonstrated, this work is the first demonstration that amino-terminal truncations of E7 do not decrease its immunogenicity (35,36,44,52).

E7Δ21-26 produced in *P. pastoris* should elicit specific T-cell responses in humans as it contains the most immunogenic E7 regions. Indeed, natural T-cell responses to E7 mostly target its central and carboxy-terminal regions, both contained in E7Δ21-26 major products (9,53,54). In addition, most of the characterized HLA-A2-restricted E7 epitopes have been identified within the E7 carboxy-terminal region (8,55). A phase I study has reported that a vaccine based on a short carboxy-terminal E7 sequence (aa 83-95) is immunogenic in HLA-A2-positive patients with HPV16 infection and high-grade anal intra-epithelial lesion; its efficacy is currently being tested (12). The only HLA-A2-restricted E7 epitope absent from the E7Δ21-26 preparation is E7<sub>11-20</sub>; however, the overlapping E7<sub>12-20</sub> epitope might be processed from the longer E7Δ21-26 product (55,56).

Collectively, our data provide evidence that E7Δ21-26 is immunogenic and generates effective prophylactic and

therapeutic anti-tumour responses in experimental mice models. The vaccine potential of E7Δ21-26 should therefore be evaluated in clinical trials.

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